

# PRACTICAL BACTERIOLOGY, HAEMATOLOGY AND ANIMAL PARASITOLOGY STITT, CLOUGH AND CLOUGH



## PRACTICAL BACTERIOLOGY, HAEMATOLOGY AND ANIMAL PARASITOLOGY

### By

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TO PHARMACOLOGY

NINTH EDITION

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### PREFACE TO THE NINTH EDITION

With each successive edition of a book of this sort, there are necessarily many additions and deletions which tend to interfere with the logical sequence of the subject matter and make the style uneven. In order to incorporate new material with the old in such a way as to give a clear and orderly arrangement, it is often necessary to rewrite the text, as we have done in the present edition.

In each new revision more space has been allotted to the interpretation and diagnostic significance of the various laboratory procedures, and in this edition we have tried to correlate still further the data obtained by these examinations with the clinical picture. The well educated technician of today is not satisfied with laboratory procedure alone, but demands a presentation of the clinical features of the disease to be investigated. Likewise the clinician and medical student obtain a better insight into the field of diagnosis if the laboratory and clinical evidence are properly coordinated. We believe that this is facilitated by bringing together in one book consideration of all the available types of laboratory procedure. Assistance in the selection of appropriate tests may be quickly obtained by consulting the Index of Useful Laboratory Procedures, page 910.

In the study of the filtrable viruses the clinical manifestations of the disease in man and experimental animals play a more important part in diagnosis than is the case in other conditions. Especially in haematology in which there has been such a rapid advance in our knowledge of these diseases and in their differentiation, a concise presentation of the symptomatology is needed, as well as details of chemical and microscopical technique. In some instances we may appear to have stressed unduly the symptomatology of certain diseases, but this has seemed advisable if the material is to be found only in the recent literature, or if, from the rare or exotic nature of the disease, it is dealt with inadequately or not at all in the standard text books.

We have continued to bear in mind the needs of the man in tropical or remote fields where he does not have access to well equipped libraries.

Those familiar with former editions will note that the chapters on Apparatus, Culture Media and Staining Methods have been put in the appendix, whereas such sections as chemical examinations of the blood and urine, kidney and liver function, and the food-deficiency diseases have been transferred to Part IV. We have tried to save space wherever possible by eliminating obsolete material and by using small type more freely. We have also omitted from the appendix the section on histological technique, and much of that dealing with the problems of nutrition, since this material is now generally available in standard text books. In spite of this the text of the present edition contains 113 pages more than the eighth edition.

On the first page, under "Classification" will be found a table based chiefly upon Bergey's Determinative Bacteriology. In Chapter I will be found a concise presentation of bacterial dissociation. The important questions of smooth and rough strains and of antigenic structure have been discussed particularly in connection with the typhoid-colon group, although referred to in the text on other bacteria.

We think that the table on "Diagnosis of Infectious Diseases by Animal Inoculation" covering the last four pages of this chapter will be of great value. In the same chapter is another table listing the diseases which may be contracted from domesticated animals. As in previous editions, this edition contains a great number of tables, but of particular value is the one on the vitamins (Chapter XLII, page 790). The general discussion of vitamins in the eighth edition covered 14 pages, whereas in the three pages of this new table, the information obtainable is more accessible and more comprehensive.

The chapter on "Filtrable Viruses" is almost entirely new, and those on the spirochaetes and the rickettsias have been rewritten. In this edition these latter organisms have been transferred from the protozoal to the bacterial section.

An entirely new chapter on the fungi has been prepared. The classification is based on Dodge's Medical Mycology. As many of these names are unfamiliar, the different diseases are taken up under the name of the disease instead of that of the parasite. Thus, under "Torulosis," page 219, there are discussions of *Cryptococcus histolyticus* and *C. meningilidis*.

The chapter on Immunity and Hypersensitiveness (page 236) has been largely rewritten, especially that section dealing with the various allergies. We are indebted to Dr. Kahn for a revision of the Kahn test and also for a brief discussion of the relative merits of the various scrological tests for syphilis, page 264. Kahn's concept of the relations of allergy to immunity will be found on page 251. The technique of the Wassermann reaction (page 277) used by the Veterans' Administration in its numerous

hospitals has been selected as a very practical and conservative method. For this we thank Dr. Matz.

In no part of the book has the revision been greater than in that devoted to haematology. In the eighth edition there were only 67 pages on haematology and 23 on the chemical examinations of the blood, as compared with 95 and 56 pages in the present edition. We have paid special attention to the technique of blood examinations and have given in detail those methods which we regard as the most useful and important. We have added two colored plates and two colored drawings from cases of leukaemia to show the detailed structure of the different types of blood cells. We have attempted to classify the anaemias on the basis of their pathogenesis, and have summarized the distinctive features of the more important types in the table on page 381.

In the chapter on Chemical Examination of the Blood we have included a discussion of disturbances of acid-base equilibrium which we hope will be helpful to those with limited mathematical training.

In Chapter XLI there is a summary of the more recent views as to the function of the endocrine glands. In this chapter both the Friedman and the Aschheim-Zondek tests for pregnancy are given. The Friedman test as outlined by Dr. Anna M. Young is given preference. We extend our thanks to her for the illustrations.

In Chapter XLII, besides the table of the characteristics of the vitamins previously referred to, will be found a rather detailed consideration of the principal food deficiency diseases. The recent facts as to cevitamic acid in relation to scorbutic conditions are outlined under scurvy, page 802.

In the appendix the sections on toxic plant products and on communicable diseases have been greatly extended. In particular we call attention to the very complete revision of the final section (Laboratory Procedures Useful in Diagnosis). We have selected those procedures which we believe are the most useful, and have listed them for each of the more important diseases. We have made this easily accessible by indexing the diseases alphabetically.

We have been fortunate in having the advice of Captain H. W. Smith as to the various changes and additions made in this edition. Other officers attached to the Naval Medical School have also given us great assistance. We here express our thanks to Commander Paul W. Wilson for very valuable notes on amoebiasis and malaria; to Commander W. W. Hall for notes in connection with medical chemistry; to Commander F. S. Johnson for advice on sanitation of ships; and to Chief Pharmacist P. T. Rees for suggestions as to chemical methods.

In particular we are indebted to Lieutenant Commander Paul F. Dickens for suggestions as to medical chemistry, and for the draft of the revision of the chapter on cerebrospinal fluid examinations. In this he had the advice of Dr. Walter Freeman of Washington, D. C.

We also extend our thanks to those of the National Institute of Health who have gone over our manuscript. In particular we are indebted to Dr. Edward Francis for extensive notes on tularaemia and brucellosis. Dr. Maurice C. Hall has been particularly kind to us in reviewing our manuscript on human helminthology, and in giving us valuable notes in this connection.

We also thank Dr. McCoy for suggestions as to the revision of the leprosy section; Dr. Dyer for notes on the typhus-like fevers; and Dr. Sara E. Branham for assistance in the revision of the meningococcus section. We likewise thank Dr. J. P. Leake and Dr. Charles L. Williams of the U. S. Public Health Service—the former for advice as to the filtrable viruses, and the latter for notes as to methods of mosquito control on airplanes.

Colonel Joseph F. Siler has given us notes on typhoid prophylaxis in the U. S. Army, and Colonel E. B. Vedder, also of the Army Medical Corps, has made valuable suggestions as to the table on vitamins.

We also have to thank Dr. Leslie N. Gay, of the Johns Hopkins Hospital, for criticism and suggestions as to the section on hypersensitiveness, and Dr. Mary V. Buell for data regarding chemical procedures used in the Chemical Laboratory of the Medical Clinic of the Johns Hopkins Hospital. We are indebted to Dr. Maxwell M. Wintrobe for numerous suggestions, particularly as to the method of determining the sedimentation rate of the erythrocytes, and to Dr. Hugh W. Josephs for the method of estimating urobilin in the faeces.

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### PRACTICAL BACTERIOLOGY, HAEMATOLOGY AND ANIMAL PARASITOLOGY

### PART I BACTERIOLOGY

### CHAPTER I

### STUDY AND IDENTIFICATION OF BACTERIA

GENERAL CONSIDERATIONS

Classification.—The classification of bacteria has always been a difficult and complex problem, and there is still a difference of opinion in regard to the classification and nomenclature of many of them. Morphological characters are of fundamental importance in all classifications, yet in the case of bacteria few structural details can be made out, and these alone do not suffice to differentiate certain organisms which are otherwise very different. It is, therefore, necessary to utilize other characteristics, such as their metabolic activities, antigenic properties and pathogenicity for animals. Although these characteristics are exceedingly important, particularly the antigenic structure, they are sometimes variable in the members of a certain species or even in a single strain under different conditions. Classification of the filtrable viruses is particularly unsatisfactory since our knowledge of them is derived largely from their pathogenic effects on animals.

### CLASSIFICATION OF BACTERIA (SCHIZOMYCETES)

- I. COCCACEAE (Family) Spherical or elliptical cells. Often flattening in plane of
  - A. Streptococceae (Tribe) Dividing in pairs to form short or long chains.
    - 1. Diplococcus: D. pneumoniae.
    - 2. Streptococcus: S. pyogenes, S. mitior, S. saprophyticus.

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- B. Neisserieae. (Tribe) Cells normally in pairs.
  - 1. Neisseria: N. gonorrhoeae, N. intracellularis, N. catarrhalis, N. flavescens.
- C. Micrococceae. (Tribe) Cells occur singly, in pairs, packets or irregular masses.
  - 1. Staphylococcus: S. aureus, S. albus, S. epidermidis.
  - 2. Gaffkya: G. tetragena.
  - 3. Micrococcus: M. luteus, M. cereus, M. subflavus.
  - 4. Sarcina: S. ventriculi.
- II. SPIRILLACEAE (Family) Cells tend to spiral curves, dividing transversely.
  - 1. Vibrio: V. comma, V. proteus.
  - 2. Spirillum: S. undula.
- III. BACTERIACEAE (Family) Rod-shaped cells without endospores.
  - A. Chromobacterieae (Tribe) Pigment-producing bacteria.
    - 1. Serratia: S. marcescens (B. prodigiosus).
    - 2. Pseudomonas. P. aeruginosa (B. pyocyaneus).
  - B. Lactobacilleae (Tribe) Long, slender, non-motile rods. Gram-positive.
    - 1. Lactobacillus. L. acidophilus, L. bulgaricus, L. boas-oppleri.
  - C. Pasteurelleae (Tribe) Non-motile, Gram-negative, bipolar-staining rods.
    - 1. Pasteurella. P. tularensis, P. pestis.
  - D. Klebsielleae (Tribe) Non-motile, plump, Gram-negative, encapsulated rods.
    - r. Klebsiella. K. pneumoniae, K. granulomatis, K. rhinoscleromatis, K. ozaenae.
  - E. Hemophileae (Tribe) Very small, non-motile cells, requiring haemoglobin or plant growth accessory substances.
    - Hemophilus: H. influenzac, H. pertussis, H. conjunctivitidis, H. lacunatus, H. ducreyii.
    - 2. Dialister: D. pneumosintes.
  - F. Bacterieae (Tribe) Gram-negative rods usually found in the intestinal tract of animals.
    - 1. Escherichia: E. coli, E. pseudodysenteriae, E. communior.
    - 2. Aerobacter; A. aerogenes, A. cloacae.
    - 3. Proteus: P. vulgaris.
    - Salmonella: S. enteriditis (Gaertner), S. schottmülleri (paratyphosus B)
       S. aertrycke (S. typhi-murium), S. paratyphi (paratyphosus A), S., morgani, S. suipestifer.
    - 5. Eberthella: E. typhosa.
    - 6. Shigella: S. dysenteriae, S. paradysenteriae.
    - 7. Brucella: B. melitensis, B. abortus, B. suis.
    - 8. Alcaligenes: A. faecalis (B. fecalis alkaligenes).
- IV. BACILLACEAE (Family) Rods producing endospores.
  - r. Bacillus (aerobic): B. subtilis, B. anthracis.
  - Clostridium (anaerobic): C. butyricum, C. welchii, C. fallax, C. oedematismaligni, C. oedematiens, C. histolyticum, C. sporogenes, C. tetani, C. botulinum.
  - V. MYCOBACTERIACEAE (Family).
    - 1. Mycobacterium (acid-fast): M. tuberculosis, M. leprae, M. phlei.
    - Corynebacterium (not acid-fast): C. diphtheriae, C. xerose, C. pseudodiphthericum.

- 3. Fusiformis: F. termitidis, F. dentium.
- 4. Actinobacillus: A. lignieresi, A. mallei, A. pseudomallei (Whitmore).

VI. SPIROCHAETACEAE: (Family)

- 1. Spirochaeta: S. plicatilis (Saprophytes)
- 2. Saprospira: S. grandis. (Saprophytes)
- 3. Cristispira: C. balbianii (Parasites in molluscs)
- 4. Borrelia: B. gallinarum, B. recurrentis, B. duttoni. B. vincenti.
- 5. Treponema: T. pallidum.
- 6. Leptospira: L. icterohaemorrhagiae, L. morsus-muris.

This classification largely follows Bergey's Determinative Bacteriology, Fourth Edition. Although this is not official, it was based upon the recommendations as to nomenclature of a Committee of the Society of American Bacteriologists. Although Bergey's Manual includes the genus Actinomyces among the bacteria, we have followed Ramsbottom and classified these organisms under the Fungi Imperfecti. Under Streptococci we have not given separate species for the haemolytic streptococci of scarlet fever, erysipelas, puerperal fever, and septic sore throat, accepting the views of Anna W. Williams that these organisms are but varieties of S. pyogenes. The type species of each genus is italicized. In a few instances in which the type species was of no medical importance, it has been omitted. The rules governing zoological nomenclature are discussed in Chapter XVI (Animal Parasitology), and the main points in regard to which botanical nomenclature differs are noted in Chapter X (Fungi).

Since the validity of any scheme of classification of bacteria depends upon a reasonable degree of constancy in morphology and in other properties, attempts have been made to minimize variations by prescribing standard conditions as to culture media, time of incubation, etc., under which they are to be grown for purposes of description. Under the influence of Koch, who insisted on the fixity of type, those which did not conform were regarded by most observers as contaminants or involution forms or were simply ignored.

Dissociation or Variation.—Marked variations may occur in some of those characteristics of a species which were formerly regarded as of fundamental significance. The variations which at present seem to be of the greatest practical significance are those commonly included in the term "dissociation." For example, if a culture of the typhoid bacillus is carried through successive generations by streaking a single colony over an agar plate, colonies of two distinct types may be found: (1) "Smooth" colonies (S form), glistening, moist, rather viscid and homogeneous, with even round borders; and (2) "Rough" colonies (R forms), with a dry, crinkled surface, a tough, granular consistency, and irregular margins. There may be colonies intermediate or mixed in type. Subcultures from typical colonies of each type usually reproduce the corresponding type. It is relatively easy to induce dissociation from a smooth to a rough type of

### STUDY AND IDENTIFICATION OF BACTERIA

colony. This may occur spontaneously in old stock cultures or may be induced by unfavorable conditions for growth, especially by adding immune serum to the medium. Bacteriophage is often potent in initiating the change. It is more difficult to secure a reversion from the rough to the smooth type, though this is possible in some cases. For example, a fixed type pneumococcus which has been dissociated will revert to a smooth fixed type, if inoculated into a mouse together with the specific carbohydrate haptene of that type. (See Pneumococcus.)

These alterations in the appearance of the colonies are accompanied by significant changes in the other characteristics of the organisms. The *smooth type* forms homogeneous suspensions with little tendency to spontaneous clumping, and shows coarse clumps when specifically agglutinated. Motility or capsule formation, if characteristic of the species, is well marked. Such organisms are commonly met with in the early stages of the acute infections, they are relatively virulent and resistant to phagocytosis, and are more effective for immunization because they contain all the antigens of the species.

The rough type may be found in convalescence or in chronic infections. These organisms form granular suspensions, they tend to clump spontaneously and form fine clumps when specifically agglutinated. They are less virulent and are susceptible to phagocytosis. They are more resistant to bacteriophage. They often lose their flagella or their capsules, and also the corresponding antigens, retaining only the somatic antigen. There may be changes in the character of the latter also. (See sections on the pneumococcus, proteus, typhoid and paratyphoid bacilli.) They are relatively ineffective for immunization. The individual cells may show changes in morphology. They may be larger or smaller than the typical smooth strains, and may grow out into filamentous or branching forms.

Dissociation in the body.—These changes have been demonstrated in the body as well as in the test tube, and it is probable that in some infections recovery depends upon the ability of the serum to dissociate or degrade the bacteria to a form which can be phagocyted. Such variations associated with alterations in virulence may explain the rise and fall of epidemics, although there is as yet very little direct evidence to support this view. These changes introduce practical difficulties into the interpretation of agglutination tests and necessitate the careful selection of strains for the production of vaccines.

Life Cycle.—Apart from dissociation, many workers have described changes in the morphology of bacterial cells which they regard as cyclogenic; i.e., as stages in a life cycle of the bacterium. Among these may be mentioned certain of the "involution

forms" which are found in young, growing cultures and which are certainly viable and capable of reverting to cells of the usual type. In the case of bacilli of the typhoid-dysentery group, the tubercle bacillus, etc., various observers have described segmentation of the cell contents with the production and liberation of minute, viable granules 0.3 to 0.5 $\mu$  in diameter, sometimes termed "gonidia." They multiply by fission.

By cultivating typhoid and dysentery bacilli under special conditions Hadley was able to induce radical changes in the cultures. When plated on agar, only minute colonies appeared, 0.2 to 0.004 mm. in diameter, visible only with a lens, which he termed "G colonies." They were made up of minute granular forms. The G colony strains are low in virulence and grow feebly. They are usually Gram-positive. After repeated subcultures they slowly revert to the original forms.

The G colony forms are filtrable, a fact which has led some to regard the filtrable viruses in general as stages in the life cycle of bacteria. The close association of the hog cholera virus with Salmonella suipestifer, for example, has been advanced to support this view. The weight of evidence is strongly against this, however, particularly in view of such characteristics of the filtrable viruses as their inability to grow except in association with living cells. The evidence for the existence of minute, viable, filtrable forms of certain bacteria seems conclusive. The development of slender rods from granular fragments of the tubercle bacillus has been observed. The exact significance of these forms, however, is not yet clear. Except for the formation of endospores, there is as yet no conclusive evidence of any truly cyclogenic forms among the bacteria.

Pleomorphism of less marked degree is not rare. A striking example in the case of *Pasteurella tularensis* is illustrated in Fig. 17, page 104. The occurrence of *mutations* (the abrupt appearance of new and permanent inherited characters) as distinguished from dissociative changes has not yet been demonstrated.

These observations, however, do not invalidate the prevailing views as to bacterial specificity.

Morphology.—Little is known as to the finer structure of the bacterial cell. It is composed of an inner cytoplasmic mass, the endoplasm, and of an outer ectoplasm which makes up the cell membrane, the flagella and the capsular material. It has been shown that in many species the chemical constitution and the antigenic properties of the ectoplasm and endoplasm are radically different. Usually the ectoplasmic structures are relatively well developed in virulent, actively growing cultures.

The presence of *nuclear material* in the endoplasm has been proved, but there is a difference of opinion as to whether this occurs in the form of minute granules scattered diffusely through the cytoplasm or is concentrated in a discrete nucleus. By special photographic methods evidence of such structures has been obtained in some large bacteria. Dobell has described minute, slender rods or spiral threads of chromatin-staining material in ordinary bacteria, which are probably nuclear in nature. The structure of the "nucleus" evidently varies in different groups of bacteria.

Endospores are formed by certain species by a process of condensation and dehydration of the cell cytoplasm. They are round or oval, highly refractile structures which stain with difficulty and resist decolorization when stained. They are said to be antigenically different from the cell substance. Their resistance to heat, desiccation and unfavorable outside environment is obviously advantageous for the continued existence of the species. It also facilitates the isolation of such organisms in pure culture, since on heating to 80°C. for 30 minutes the spores survive whereas other forms are killed. Spores are formed, however, only in young, actively growing cultures under suitable

environmental conditions. Oxygen is required, even for anaerobes (Zinsser). The anthrax bacillus, for example, will not form spores at temperatures over 43°C., or in intact carcasses of animals (in the absence of oxygen). These facts and the observation that the rate of growth in certain species is accelerated after spore formation suggest that the process also serves as a means of rejuvenating the activities of the cell.

The capacity to form spores, the position of the spore in the cell, (central, terminal or intermediate), and the relative diameter of the spore and the cell are useful points in classification. The only spore-formers of medical interest are the anthrax bacillus and a few anaerobes: the bacillus of tetanus, of botulism, and the group causing gas gangrene and malignant oedema.

Reproduction.—The usual method of reproduction of bacteria is by binary transverse fission. Exceptionally, other methods have been observed. The formation of buds or of branching filaments which give rise to cells which multiply by fission in the usual way has been described in many species and is especially characteristic of the diphtheria bacillus. The formation of intracellular "gonidia" has been described. The formation of endospores is important in the life cycle of some species, but it does not lead directly to an increase in the number of individuals. Several observers have reported structures which they interpreted as evidence of conjugation and sexual reproduction, but this explanation is not generally accepted.

Food Requirements.—Bacteria contain no chlorophyl. Except for some of the sulphur bacteria energy for growth must be obtained by oxidation or other chemical degradation of materials in the fluid medium containing them. Their requirements differ extremely. For some species these are satisfied by the meager contaminations present in distilled water, while certain pathogens grow only under highly artificial conditions in media containing blood or tissue fluids. The filtrable viruses and the rickettsiae are still more exacting in that they will grow only in association with living cells. All species of medical interest require organic material of some sort in the medium as a source of nitrogen as well as energy.

In addition to the carbon, nitrogen (as amino-acids or peptone) and mineral salts which enter into the chemical composition of the bacterial protoplasm, some species (such as Pfeiffer's influenza bacillus) also require certain accessory growth factors which probably function as vitamins. The discovery by Lloyd that these substances may be adsorbed by cotton or paper through which media are filtered was of great practical importance in improving the quality of culture media. Little is known as to the nature or mode of action of such substances. They may be obtained from a variety of sources such as fresh blood, tissues, beef infusion or potato juice.

Oxygen Requirements.—Most bacteria depend for their source of energy on the oxidation of organic materials. Aerobes utilize the free oxygen in the medium. Obligate anaerobes must derive their oxygen from other sources: from reducible substances in the medium or from the products of cleavage of carbohydrates or proteins. It has been suggested that the inhibitory effect of free oxygen on obligate anaerobes is due to the toxic effect of peroxides which these organisms are unable to split. Such organisms may be made to grow in the presence of some free oxygen by adding to the medium a little sterile tissue or unheated potato which contain a catalytic ferment. To a limited extent anaerobes may derive energy from such processes as the alcoholic fermentation of sugars or the cleavage of protein, in which no oxidation occurs. Viruses may also be aerobic or anaerobic. Most aerobes are facultative anaerobes, but some, such as the pneumococcus, the plague bacillus and the tubercle bacillus, require abundant free

oxygen. Some organisms, particularly on isolation, require a reduced oxygen tension and will not grow under either aerobic or strict anaerobic conditions. This is most easily secured by using deep tubes of agar containing pieces of sterile tissue at the bottom of the tube. Colonies appear only in some intermediate zone of the agar column.

Metabolic Products.—In the course of their active growth bacteria produce a great variety of substances. Some of these are enzymes which play an essential part in the nutrition of the organism. Proteolytic enzymes are formed by a great variety of bacteria. These may be demonstrated by testing the capacity of a culture to liquefy gelatin, coagulated blood serum or egg white, and are important in classification. The capacity to split carbohydrates and to ferment various sugars and alcohols also depends on special enzymes. As a rule these activities are fairly constant and specific, and are important in identification, particularly of members of the typhoid-dysentery group. The activities of some of these bacterial enzymes are of great industrial importance, such as the alcoholic fermentation of sugar, the oxidation of alcohol to acetic acid and the cleavage of corn starch with the production of butyl alcohol and acetone.

As a result of these metabolic activities, various by-products accumulate in the culture media, such as acids, alcohols, peroxides, amino-acids, and other protein decomposition products, which inhibit the growth and eventually bring about the death of the organisms which produce them. The by-products of one species usually form the pabulum for another, until under natural conditions the dead bodies and refuse of plant and animal origin are completely broken down into simple substances (CO<sub>2</sub>, nitrates, water) which can be utilized by chlorophyl-containing plants and resynthesized into materials which can serve as food for animals. Bacteria thus help to maintain the "circulation" of carbon and nitrogen in usable form between the plant and animal kingdoms.

Toxic Substances.—Pathogenic organisms also form various substances which are injurious to their host and which doubtless protect them more or less effectively from the antibacterial action of the defensive forces of the host. Among these are the specific soluble toxins, secreted by certain bacteria and liberated into the surrounding medium. Another type is met with in the specific soluble substances contained in the capsular material of pneumococci and Friedländer bacilli. This appears to endow the organism with the capacity to resist phagocytosis and invade the tissues. Failure to form capsules is associated with loss of virulence. Bacteria form other "aggressive" substances which appear to be an integral part of their protoplasm and are liberated only on disintegration of the bacterial cell (the endotoxins). These are complex mixtures and may contain two or more distinct fractions, each possessing antigenic powers and each capable of stimulating the production of antibodies and of allergic reactions in the host, specific for itself. These antigens may be restricted in distribution to the one species of bacterium producing them, or one or more of them may be found also in related species or even in unrelated organisms, as in the case of the rickettsiae and Proteus vulgaris. antigenic content of a strain may be altered if the culture becomes dissociated. These complex relationships have been studied especially in the typhoid-dysentery group and will be discussed in that section.

Factors Determining Infection.—In general the occurrence of infection depends upon the balance between the invasive powers of the organism

### STUDY AND IDENTIFICATION OF BACTERIA

and the defensive forces of the host. The various means by which the host resists infection are discussed in a later chapter. If infection is to be established the invading organisms must possess an adequate degree of virulence. It is necessary that the number of invading organisms be adequate. Although in exceptional instances it is possible for a single organism to infect, as in the case of highly virulent anthrax bacilli or pneumococci in the rabbit, as a rule an appreciable number are required, and if the dosage is insufficient the invading organisms will be destroyed. On the other hand a massive dose may infect even though the virulence is relatively low or the resistance of the host high. The invaders must also reach a suitable portal of entry into the body. Thus infection may be brought about readily if the virus of poliomyelitis reaches the olfactory mucosa or the typhoid bacillus reaches the intestine, although neither would be likely to penetrate the skin. On the other hand infection with a virulent haemolytic streptococcus would be more likely to occur through a skin abrasion than through the gastrointestinal tract.

A recognized fact in epidemiology is that in the spread of an epidemic certain individuals seem to escape infection, notwithstanding intimate exposure; some contract the disease in a mild form, whereas others suffer severely or die in the course of the epidemic. As regards the nature of such individual immunity there is little exact information, but it furnishes a great field for investigation.

As stated by Theobald Smith, the cooperation of two parasites (symbiosis) is to be thought of in the spread of epidemics, and this field is an important one. The best known example of the symbiosis of bacteria is the association of the spirillum and fusiform bacilli of Vincent's infection, and probably of anaerobic streptococci also in severe cases. Recognition of the association of the hog cholera bacillus and the filtrable virus was an outcome of Dorset's work. Koch's postulates were satisfied as to the relation of the hog cholera bacillus to the disease, but later investigations disclosed a viral etiology. This may also be a synergistic relation. Kruse has emphasized the importance of "begleiter" (companion) parasites, and the possibilities in this field of research are now recognized. Man may have ever present one parasite which awaits the "genius epidemicus" of a companion parasite transmitted from other human or animal sources. The study of the connection between the influenza of swine and that of man is now being actively pursued. In both there is the frequent association of an organism of the Pfeiffer type. Shope, in his work with swine influenza, has shown that a bacillus of this type as well as a virus is required (symbiosis).

Adaptation.—The severity of the symptoms that accompany an infection depends in some cases upon the capacity of the by-products of the organism to injure the host on the one hand, and the effectiveness and promptness of the defensive forces of the host in limiting multiplication of the organisms and neutralizing their toxic products on the other. In other cases, in spite of the successful invasion of the host and continued growth of the organisms, the host suffers little or not at all. It is obviously advantageous to the parasite as well as to the host to live and multiply in the tissues of the host without seriously injuring the latter. Such an adaptation of the host and the parasite to each other, so that virtually a state of commensalism exists, is believed to be the result of a phylogenetically long period of association. An example of such an adaptation is found in infection of the rat with *Trypanosoma lewisi* which appears to be quite harmless to these animals. This state is approached in the case of the virus of simple herpes in man.

The bacteria constituting the "normal flora" of the upper respiratory passages and the intestinal tract behave as harmless commensals as long as they do not penetrate the mucous membranes. Some of them, however, may become pathogenic if they succeed in gaining a foothold in the tissues. The adaptation of bacteria to the host rarely reaches such a point that they can grow in the tissues without causing injury. In some individuals tubercle bacilli or the treponemata of syphilis may live in the tissues and even proliferate to a limited extent without causing appreciable injury. A few individuals of more virulent species may persist for long periods in the tissues in a latent but viable state, particularly in the lymph glands and spleen or in inflammatory scar tissue. Such dormant infections, however, constitute a constant danger to the host. Even in the case of comparatively avirulent organisms like Streptococcus viridans, which are relatively well adapted to man as a host and rarely cause acute symptoms, the persistence of living organisms in the tissues (focus of infection) often results in serious chronic injury, whether it be from bacterial toxins or chronic allergic reactions to the bacterial protein.

Propagation of Infections.—The mechanism by which an infectious disease is propagated in a community brings up many complicated and interesting problems. Much depends upon the characteristics of the organism; its portals of entry and exit; how long it persists in the host after recovery; how long it retains its virulence; whether it can survive unfavorable conditions and even multiply outside the body; and whether it can infect other mammals or arthropods. The details differ and must be studied individually for each different species of organism. We can cite only a few examples.

In some cases such as *Treponema pallidum* the organism is an obligate parasite, it infects no other animals under natural conditions, it can not survive for any appreciable time outside the body, and it is transmitted only by direct physical contact with an infected individual, or by coming within the range of droplet infection (as in measles).

In the case of diphtheria and pneumococcus pneumonia essentially the same is true, but conditions are complicated by the fact that in some cases the organisms may be present in a virulent state in convalescent or healthy carriers, who constitute a more

dangerous, because unsuspected, source of infection. A carrier state may exist in some virus diseases, such as poliomyelitis and herpes in man, psittacosis in parrots and even rabies in the vampire bat. Eventually such strains in carriers may become dissociated and lose their virulence and infectivity. On the other hand it is probable that such dissociated strains, if they can maintain themselves on the mucous membranes as saprophytes, can regain their virulence under special conditions and may then infect or reinfect the host.

In the case of the typhoid and dysentery bacilli infection by direct contact is possible but unusual. These organisms are able to survive for considerable periods outside the body under favorable conditions. Leaving the body chiefly in the facees (or urine) of patients or healthy carriers, they may contaminate the hands or fomites, or are readily disseminated in the soil or in water in a condition to infect new hosts if conveyed mechanically to their alimentary tract. These organisms do not usually multiply outside the host, except in milk or other liquid foods. These materials are then extremely dangerous because of the massive dose of the infecting organisms which they convey. These organisms do not naturally infect other animals, and arthropods play only an incidental and purely mechanical rôle in their transmission.

Another group of organisms is exemplified by the plague bacillus which naturally parasitizes rats and various other rodents. These animals constitute a natural reservoir of infection from which sporadic cases and, periodically, great epidemics in man arise. Infection is commonly conveyed from rat to man by the rat flea, within which the bacilli multiply actively. In the case of bacteria, however, this is incidental; no bacterial infection is known in which an arthropod is required to complete the life cycle of the organism. Man may also be infected through the skin by direct contact or by ingesting the organisms. In the case of tularaemia, rabbits and other wild rodents constitute the natural reservoir of infection, and man is infected by the bites of infected ticks, deer flies and probably other arthropods, as well as by direct contact. The arthropod host, however, is not obligatory for the existence of the parasite, as in the case of Glossina and Trypanosoma gambiense.

In other cases the infections are more distinctively animal diseases, and man is only occasionally and incidentally infected. Such are glanders, transmitted by direct contact, and brucellosis, transmitted usually by infected milk. In the case of anthrax and foot-and-mouth disease, the infective agents (a sporulating bacillus and a filtrable virus, respectively) are very resistant to desiccation and other injurious agencies outside the body, and man may be infected by contact with contaminated soil or barn-yard objects.

Finally, there is a large group of viral, protozoal and helminthic infections in which one or more other species of animals (usually arthropods) are required for completion of the life cycle of the parasite. As a rule a sexual cycle occurs in one host (the definitive host) and an asexual cycle in the other (the intermediate host). Usually there is a more complete adaptation of the parasite to the definitive host (which suffers little or no injury from the infection and therefore assures the survival of the parasite species) than to the intermediate host. It is probable, therefore, that the parasite was limited originally to the definitive host, and that the capacity to attack the intermediate host is phylogenetically a recently acquired character.

Typical examples are malaria, in which man is the intermediate host and Anopheles mosquitoes the definitive hosts; and yellow fever, in which the vectors, Aedes mosquitoes, behave like definitive hosts. In the case of filariasis, man is the definitive host and

Culex mosquitoes the intermediate hosts. Hereditary transmission does not occur in these mosquitoes. In malaria there is no mammalian reservoir, but in yellow fever certain species of monkeys and other mammals may be naturally infected.

In the case of some organisms in which no such life cycle is known to occur, arthropods constitute a permanent reservoir of infection and presumably represent the original host of the parasite. This appears to be the case with African Tick Fever, the parasite of which (Borrelia duttoni) is found in the tick, Ornithodoros moubata, and is transmitted through the egg to successive generations of ticks. The Rickettsia of Rocky Mountain spotted fever seems to have undergone a series of adaptations to successive hosts. It is thought that the organism was first a plant parasite; then became adapted to the mites which fed on the plants; then to the rodents to which the mites had become adapted; and finally to the ticks which infest the rodents. The organisms are transmitted hereditarily in the ticks. There is some reason to believe that Leishmania and Trypanosoma were originally insect parasites which have become secondarily adapted to man and other nammals. These views, however, are not universally accepted.

Infectious agents may become adapted to new tissues as well as to new hosts. Thus if the freshly isolated virus of yellow fever or Rift Valley fever is injected intraperitoneally into monkeys, it causes visceral lesions but not encephalitis. If the virus is carried through a series of mice by intracerebral injections (causing encephalitis) and is then injected intraperitoneally into monkeys, it will no longer cause visceral lesions, but will cause encephalitis if the brain is first injured, or if the virus is injected intranasally or intracerebrally. The virus has become neurotropic, although it is not changed in its serological reactions. Many of the filtrable viruses exhibit a remarkable capacity for adaptation both to new tissues and to new hosts.

### METHODS OF ISOLATION

Isolation in pure culture is indispensable for the study of bacteria.

Stroked Plates.—Isolation may be accomplished by suitably diluting the material for culture and stroking the material over the surface of solidified agar plates. It is advisable first to examine a stained film of the material to be cultured to determine approximately the number of organisms present and the degree of dilution necessary in a tube of sterile salt solution or broth. A loopful of the material, diluted if necessary, is placed on the surface of an agar plate near one margin. As a spreader one may use a small sterile cotton swab, or better, a fine glass rod or platinum wire, the end of which is bent at a right angle about two cm. from the tip. Dilution is accomplished most rapidly by using small spreaders. In streaking plates with a glass rod several plates may be required to obtain discrete isolated colonies, while with a platinum wire the same result may be obtained with only two or three plates. The spreader is sterilized by heat and cooled, and with the bent tip the material is stroked over the surface of two or three agar plates in a series of parallel sweeps without sterilizing or re-inoculating the spreader. The weight of the handle of the spreader must be supported by the fingers so that the surface of the medium is not roughened.

As a rule, the distribution will be such that discrete colonies are obtained after incubation on one of the plates, sufficiently separated from each other so that the characteristics of each type of colony can be recognized and individual colonies "fished" and subcultured for further study and identification. This can be done most conveniently

by placing the plate on the stage of a dissecting microscope and touching the colony with the tip of a platinum needle while looking through the lens. As the needle is about to touch the colony, its point comes clearly into focus. It is well to support the right hand with the left in order to steady the needle. If the colony is large enough a second particle may be fished from it and rubbed up in a small loopful of water on a coverslip, examined for motility, and then spread and stained by Gram's method. Each colony examined should be clearly marked and numbered on the plate with a wax pencil and the same number affixed to each slide or culture tube subsequently used in studying this organism. It is important to wait at least 48 hours (in the case of *S. viriduns* or *Brucella* seven days) for the development of colonies before discarding the culture.

Some prefer to spread the material evenly over the entire surface of the plate, revolving the plate while drawing the spreader back and forth; then without sterilizing the spreader to stroke it successively over two or more additional plates in a similar manner. To economize on materials the two halves of one or two plates may be stroked successively. A satisfactory procedure is to put a loopful of diluted material on the left half of the plate and then without recharging the loop to touch the right half of the plate with the loop. Then press the bent end of a sterile spreader into the middle of the plate, dividing it into two portions. Next spread the small amount of material evenly over the right half of the plate, and then spread the loopful over the left half.

For routine examination of most types of material from patients blood agar plates should be used, or suitable special agar media, rather than plain agar. For faces we use Endo, bismuth sulphite, or Teague's medium. Urine, if heavily infected, should be diluted. If not, spread one drop on one half of the plate and five drops on the other half.

Poured Plates.—From one to several loopfuls of material may be mixed with a tube of melted agar, one or more loopfuls transferred from this tube to a second tube and mixed, and a similar quantity transferred from this to a third tube. The contents of each tube are then poured into Petri dishes, allowed to harden, and incubated. A serious drawback to this method is the fact that most of the colonies are "deep," below the surface of the agar, and such colonies do not show adequately the specific differences evident in the surface colonies. This procedure is rarely used now except when serial dilutions of material are to be plated for the purpose of counting colonies.

In using melted agar for plating it must be remembered that agar solidifies at a temperature slightly below 40°C. and does not melt until the temperature is raised nearly to the boiling point. Since the organisms to be cultivated may be injured by temperatures over 44°C., it is necessary to work carefully and quickly to avoid premature solidification of the agar. Gelatin plates are of little use in medical bacteriology, since most pathogens require a temperature at which this medium is liquefied.

Agar Slants.—Instead of using plates, material on a platinum loop may be stroked successively over the surface of four or five agar or blood agar slants, or successive dilutions may be made in the water of condensation of several blood agar tubes. The water of condensation is then made to flow once over the surface of the slants, and the tubes are incubated. Discrete colonies can be obtained which can be examined with a lens and fished without difficulty, although not so conveniently as from plates.

Stock Cultures.—For the maintenance of stock cultures stab cultures or fluid cultures are preferable to slants since they require much less frequent transfer and are less easily damaged if packed for shipment. Many species will remain viable for months if the culture is kept frozen.

Barber's Technique for Isolating a Single Bacterial Cell.—This method is very valuable in securing indisputably pure cultures of bacteria, especially anaerobic spore-bearers. It is especially important as a preliminary step in the study of dissociation or possible mutational changes. Much practice is required to master the procedure, and the reader is referred to Barber's article for detailed technique (Philippine J. Science, 1914).

In brief outline the procedure is as follows: A glass box about 7 by 3 by 2 cm., open at one side and without a top, is fixed in the mechanical stage of the microscope. A large cover slip, 60 by 35 mm., is lightly coated with sterile petrolatum, and a drop of culture, considerably diluted, is deposited on the greased surface. The cover slip is then placed over the open top of the glass box, greased side down. The lens is then brought into focus on the surface of the drop. A firm capillary pipette, bent at right angles near the tip, with the tip drawn out so as to present a fine lumen, and with the other end attached to a delicate rubber tube for making suction or pressure, is introduced through the open end of the box and the tip brought into focus. The pipette may be guided by hand if one is very skilful, but it is usually necessary to use a mechanical device by which the pipette is held and moved under observation through the microscope. The tip of the (sterilized) pipette is brought up to touch the surface of the drop near the organism desired, and a minute drop of fluid drawn into the tip, either by capillary attraction or if necessary by suction. The pipette is then moved under another portion of the cover slip, the drop of fluid blown out onto the cover slip and inspected. A second sterile pipette is then introduced and the procedure repeated until a single organism is obtained.

### METHODS OF IDENTIFICATION

The most useful points to be noted for the purpose of identifying a culture after isolation are the following:

Morphology.—Fundamental points are the shape, size, and arrangement of the organisms; the formation of packets, tetrads, chains or palisade-like clusters, and the occurrence of polar bodies or metachromatic granules. In some cases (as plague bacilli on salt agar) characteristic involution forms occur.

Motility.—A young (twelve to eighteen-hour-old) culture should be used. A small drop is placed in the center of a cover slip, and this is mounted over the concavity of a hollow-ground slide. If this is not available, make a ring of vaseline on an ordinary slide, put the drop in the middle of the ring and lower a cover slip onto it. This prevents evaporation and the production of fluid currents. An eighteen hour growth on an agar slant may be touched with the tip of a platinum needle and this rubbed up in a drop of water or salt solution which is then mounted in a similar way. Examine with the light much cut down with the diaphragm. First focus on the edge of the drop with the 25 in. objective and then examine with the 16 in. objective (not with the oil-immersion lens).

If truly motile, some of the bacteria will show progressive motility in various and diverse directions, and will move away from the place where they were first observed. This must be differentiated from motion due to currents in the fluid, which is characterized by the swarming of all the bacteria in the same direction, at varying speed; and from Brownian movement, a rapid oscillating motion of small amplitude, which may be observed with any minute inert particles in a liquid. Only a few of the organisms in the field may show true motility. Occasionally a strain may show little or none imme-

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diately after isolation but show active motility after several subcultures in broth have been made. Motility may be lost if a culture becomes dissociated.

Flagella.—The number and the arrangement of the flagella in the case of motile organisms is also sometimes distinctive. An organism may have a single flagellum at one pole (monotrichous); flagella at each pole (amphitrichous); or many flagella all around them (peritrichous). The cholera vibrio, a monotrichous organism, moves at a rate of 0.030 mm. per second, while the typhoid bacillus which is peritrichous has a rate of movement about half as great—0.018 mm. The technical difficulties in staining flagella are so great that the procedure is seldom used for routine diagnosis.

Spore formation has been discussed (p. 5).

Gram's stain is of fundamental importance in differentiation. Young cultures not over 24 hours old must be used and the staining solutions must be efficient. A strain may lose its capacity to retain the stain after long continued cultivation on artificial media. Churchman showed that the growth of Gram-positive organisms is inhibited by a minute amount of gentian violet in the medium.

Practically all pathogenic cocci are Gram-positive except the gonococcus, the meningococcus and *N. catarrhalis*. Practically all pathogenic bacilli are Gram-negative except the spore-bearers, the acid-fast bacilli and the diphtheria group.

Special staining reactions (described in the special sections) are important in the recognition of certain organisms; notably capsule stains (pneumococcus, Friedländer bacillus), acid-fast stains (tubercle bacillus), and stains for polar bodies (diphtheria bacillus).

### CULTURAL CHARACTERISTICS

Single Colonies.—The characteristics of individual colonies on agar or blood agar plates are important. The points to be noted are the size and shape, the degree of opacity, the character of the margin, of the surface (smooth or rough), the texture and consistency (moist and viscid or dry and tenacious), and the presence or absence of pigment. On blood agar the presence of haemolysis or of a greenish discoloration of the blood around the colony is very important in the differentiation of streptococci.

Broth cultures are useful in identification chiefly for tests for motility. Look for turbidity, sediment, and pellicle or stalactite formation.

Potato cultures are useful only in special cases, particularly in stimulating pigment production.

Litmus milk cultures have been extensively used, but the information they yield is better obtained by other methods, particularly fermentation tests. Note successive changes in reaction, coagulation, liquefaction, and gas formation.

Gelatin.—Liquefaction of gelatin is an important means of differentiation of bacteria in general, but is relatively unimportant in the study of pathogenic organisms except the cholera and proteus bacilli (bacteriology of water). If plates are used a room-temperature incubator (20°C. to 22°C.) is required. If one is available, deep stab cultures may be made, and the shape of the liquefied area noted as well as the presence or absence of liquefaction. If a low temperature incubator is not available, or if the pathogens do not grow at low temperatures, the tube should be incubated at 37°C., and then immersed in ice water. Failure of the gelatin to solidify indicates that the organism is a liquefier. Liquefaction is effected by an extra-cellular ferment, gelatinase, which is present in bacteria-free filtrates of liquefying organisms.

Fermentation of carbohydrates is of great importance in the identification of many organisms, particularly of the typhoid-dysentery group. For ordinary routine work glucose and lactose are chiefly used, but for special studies many other sugars, alcohols and other carbohydrates are also employed. The occurrence of fermentation is indicated by the production of acid and in some cases of gas also. The most satisfactory medium for demonstrating fermentation consists of a 1% solution of the carbohydrate in serum-water together with an indicator (Andrade's reagent), sterilized in fermentation or Durham tubes. Gas production can also be demonstrated by making deep stab cultures in glucose (or other) agar tubes or better by inoculating a tube of such agar which has been melted and cooled to 42°C. Fermentation tubes are unnecessary for the study of pathogenic cocci, as none of these organisms form gas.

Indol.—The production of indol (a decomposition product of tryptophane, one of the amino acids of protein) is characteristic of bacteria which are active in causing the decomposition of protein ("putrefaction"), such as the colon and proteus bacilli. The presence of carbohydrate in the medium may inhibit indol production. The indol is not broken up by these organisms and may be recognized by adding a few drops of  ${\rm H}_2{\rm SO}_4$  in the case of cholera, a nitrate-reducer (cholera red), or of a dilute solution of a nitrite together with the acid, as in the case of the colon bacillus. The variability of indol production lessens the diagnostic significance of the reaction. The tests for indol are described on page 842.

Special Media.—The selective bacteriostatic action of certain anilin dyes is often utilized in the isolation of certain bacteria, which is difficult and uncertain with ordinary media. They are also of some diagnostic value. Among them may be mentioned the media of Petroff (gentian violet), Endo (basic fuchsin) and Teague (eosin and methylene blue). The specific action of these dyes is not due to their color but to the presence of certain molecular groups which may be colorless. Other substances which exhibit neither color nor fluorescence may have a similar action.

Oxygen Requirement.—This is not of much diagnostic value since most pathogens are facultative anacrobes. The most important obligate aerobes are the organisms of plague, tuberculosis, and glanders, the pneumococcus and the gonococcus. The obligate anacrobes include the bacilli of tetanus, botulism, malignant oedema, and gas gangrene, the treponemata and leptospirae. An important point in the recognition of Brucella abortus is its inability to grow on isolation except in an atmosphere of 5 to 10% CO<sub>2</sub>.

Serological Reactions.—The complete identification of some species of bacteria, such as the group of dysentery bacilli, or the types of bacteria within a species, such as the pneumococcus, depends upon serological reactions. This method depends upon the fact that when bacteria are injected into a suitable animal the serum of the animal acquires the property of reacting specifically in various ways with the species of organism injected but not, as a rule, with other species. This property depends upon the development in the inoculated animal of specific protective substances or antibodies. The more important manifestations of this property are. (1) Agglutination of the organisms. (2) Precipitation of soluble products of the organism in fluid media. (3) Stimulation of phagocytosis of the organisms in the presence of leukocytes. (4) Fixation of complement when the organisms or their products are mixed with their specific sera. (5) Bacteriolysis of the organisms. (6) Neutralization of toxins produced by the bacteria. (7) Ability to protect animals from otherwise fatal inoculations of the bacteria due to one or more of these properties and possibly other unknown activities.

While these activities are, in general, specifically limited to the species of bacteria used, they may be exerted to a greater or lesser extent upon biologically related species (group reactions). These group reactions are usually quantitatively less marked and, therefore, evident only in higher concentrations of the serum than with the specific organism. For example, the serum of an animal inoculated with typhoid bacilli may also agglutinate, to a less extent, paratyphoid bacilli. Occasionally, however, the difference in the degree of the serum action on the two species is slight, and it may be necessary to resort to agglutinian absorption tests or other special procedures (see Chap. XII). These procedures are further complicated by the changes in the antigenic properties of some of the organisms due to dissociation. These special individual changes will be discussed in the chapters dealing with those organisms in which such changes have been studied.

Conversely, the behavior of the serum of an individual with an infectious disease may be tested, similarly, with known species of bacteria or their products in order to determine the etiology of the infection; for instance, the agglutination reactions in typhoid fever and brucellosis.

THE RÔLE OF DOMESTICATED ANIMALS IN THE TRANSMISSION OF DISEASE

Prior to and subsequent to the Middle Ages it was frequently claimed that human epidemics had as origin epizootics in other animals. Modern epidemiological research has established a foundation for this view in a few instances, but, as a rule, disease transfer from domesticated animals is of minor importance when contrasted with the diseases communicated by man to man. In fact, one of the extraordinary findings of immunology is the lack of susceptibility of one kind of animal to the diseases of other species. This resistance even extends to certain varieties of a species.

Adaptation of parasites.—As regards the possibility of the adaptation of parasites of other animals to man, it is interesting to note that the two domesticated animals with which man has been most intimately and for the longest time associated (horse and dog) are much less likely to communicate disease to him than animals domesticated at later periods—as swine or cattle. The idea is frequently advanced that many of our parasites have evolved from plant or arthropod sources, but Theobald Smith states: "It is highly doubtful that new diseases or host-parasite combinations are in progress of development from saprophytic or free living forms. More probable is the modification of existing parasites through aberrant parasitism in alien hosts, or the advance of superficial mucous-membrane forms into invasive types."

The tables on pages 17 and 18 give an idea of the importance of the epidemiology of diseases contracted by man from domesticated animals.

### ANIMAL EXPERIMENTATION

Animal inoculation is extremely important as a means of isolating and identifying many pathogenic organisms. The susceptibility of a certain species of animal and the type of disease produced is frequently essential

### DISEASES OF ANIMALS TRANSMITTABLE TO MAN

Disease	Infective source	Remarks
Actinomycosis. (See page 227.)	Cattle (lumpy jaw).	Epidemiology of cattle and human infection controversial (symbiosis?). Evidence of human infection from cattle insufficient.
Anthrax. (See page 56.)	Cattle, horses, sheep.	In cattle, hides; in horses, hair for shaving brushes; in sheep, the wool.
Brucellosis. (Sec page 100.)	Goats, cattle, swine.	Milk main source of infection from cows and goats. Carcass of infected hog at abattoir. Bovine strain least virulent.
Erysipeloid (rotlauf).	Swine?.	Contact with bone or carcass of hog with swine erysipelas (usually of hand). Not serious. Actinomyces thuillieri (Erysipelothrix rhusiopathiae).
Foot and mouth disease. (See page 177.)	Cattle, hogs, sheep, goats.	Milk or saliva of infected cow usual cause of human infection (children). Symptoms in man very mild.
Flukes. (See page 465.)	Cats, sheep.	Cats—Opisthorchis felineus. Sheep—Fasciola hepatica.
Glanders. (See page 91.)	Horse.	In man an acute and chronic type of disease. Acute resembles pyaemia; chronic, ulcers and lymphatic gland involvement.
Hydatid disease. (Sec page 481.)	Dog.	Ingestion of Echinococcus granulosus ova. Close association with infected dogs makes possible ingestion of dog faecal material.
Milk sickness. (See page 899.)	Cow.	Milk from cows which have fed on white snake root or rayless golden rod.
Plague. (See page 110.)	Rat.	During plague epizootic in rats, rat fleas take up plague bacilli in rat's blood. Feeding on man transfers infection.
Psittacosis. (Sec page 193.)	Parrot, parrokeet, love bird.	Virus disease. Diarrhoea in parrots, pneumonia in man.
Rabies. (See page 184.)	Dog.	Bite of rabid dog.
Rat bite fever. (See page 148.)	Rat, cat.	Bite of infected rat, or cat which has recently eaten an infected rat. L. morsus-muris.
Rift valley fever.	Lambs.	Contact with lambs sick with this virus disease may produce a dengue-like disease in man.
Ringworm.	Cats, dogs, horses, mice.	Microsporum canis and M. felineum frequent in children—scalp and glabrous skin. Mouse favus (Achorion) may produce body lesions in man.

Disease	Infective source	Remarks
Salmonella (food poisoning). (See page 123.)	Cattle, rats.	Pood poisoning from meat of infected cattle or contamination of food by faeces of in- fected rats.
Septic sore throat. (See page 31.)	Cow.	Human streptococcus (beta type) infects udder and milk. Streptococcus of chronic mastitis of cow is an alpha or alpha prime type (S. mastitidis).
Swine influenza. (See above.)	Swine?.	Influenza of swine and man identical or closely related (symbiosis of virus and bacillus).
Tape-worm. (See page 478.)	Cattle, hogs (fish).	T. saginata—cattle, T. solium—hog, D. caninum—dog, D. latum—fish.
Trichinosis. (See page 493.)	Hog, rat.	Ingestion of raw or insufficiently cooked infected pork meat. Rats eat infected pork meat at abattoir and are later eaten by hogs.
Tuberculosis. (See page 73.)	Cattle, fowls.	Bovine tuberculosis mostly in children (un- pasteurized milk). Tuberculosis of lungs (adult) o %, children 6 %. Bones, joints, glands (adult) 10 %, children 25 %. Bovine strains cause about 5 % of tuberculous men- ingitis. Fowl tuberculosis is very rare in man.
Tularaemia. (See page 104.)	Rabbits.	Fly or tick transmission from rabbits (other animals much less important). Inoculation from handling infected rabbits. Eating insufficiently cooked rabbit.
Typhus-like fevers. (See page 159.)	Rat, mouse (dog).	Endemic typhus, rat flea. Tsutsugamushi, mouse mite. Boutonneuse fever and Rocky Mountain spotted fever (Eastern type), the dog tick.
Weil's disease. (See page 145.)	Rats (rat fomites).	Food contaminated with rat urine (leptospira).

in diagnosis. It is the only means of isolating and identifying the filtrable viruses and of determining the presence of antiviral antibodies.

The experimental animals most frequently employed arc, in the order of their usefulness: the white mouse, the guinea pig, the rabbit and the white rat. Monkeys are indispensible for some purposes but are too expensive for routine work. Other animals are required for special purposes, such as the ferret for the virus of the common cold. For experimental work on vitamins A, D and E, the rat is used; for B, the pigeon; for C, the guinea pig. For D, young puppies are also used.

Methods of Inoculation. 1. Cutaneous.—The material is rubbed thoroughly over a shaven area with a glass rod.

2. Subcutaneous.— After clipping the hair and disinfecting the skin the material is emulsified and injected with a syringe and hypodermic needle of suitable size. Tissue may be placed in a pocket made by incising the skin and stripping back the adjacent subcutaneous tissues. The incision should be sealed with collodion.

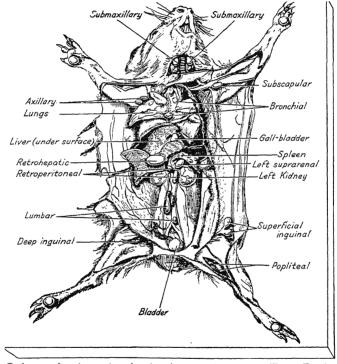


Fig. 1.—Cadaver of guinea pig, showing important organs. (From Eyre's "Bacteriological Technique.")

3. Intravenous.—In rabbits the marginal ear vein is used. This can be distended by applying either hot water or xylol. In rats or mice a vein at the base of the tail is used. In guinea pigs it is usually necessary to expose the jugular vein or to make the injection directly into the heart under anaesthesia. The needle is inserted in the second right interspace near the sternum, with the tip directed backward, downward and toward the left. Exert moderate suction with the syringe and vary the depth to which the needle

is inserted until blood enters the syringe freely. Then inject. The apex beat may be located by palpation and the needle plunged directly into the ventricle at this point.

- 4. Intraperitoneal.—After shaving and disinfecting the skin of the abdomen introduce the needle obliquely through the skin in the median line and then vertically through the muscle until the tip is within the peritoneal cavity. To avoid puncturing the gut it is desirable (particularly with rabbits) to hold the animal with the head down so that the intestines gravitate away from the needle.
- 5. Intracerebral. Rabbits.—The head is shaved and the skin sterilized. Under ether anaesthesia the skull is exposed by an incision midway between the eye and the medial ridge at the junction of the occipital and parietal bones. A small trephine opening is made and the injection carried out by means of a 1 cc. syringe with a  $^{1}4$  inch needle. Mice. Under ether anaesthesia the skin is depilated and sterilized. A fine-bore needle attached to a 1 cc. syringe is forced through the skull at a point just posterior and lateral to the vertex, to a depth of about  $\frac{1}{2}8$  inch. The maximum volume to be injected is  $\frac{1}{2}9$ 0 cc.

### CULTURING MATERIAL OBTAINED AT BIOPSY OF NECROPSY OF MAN OR ANIMALS

It is customary to sear with a heated knife or spatula a spot on the surface of the organ from which cultures are to be made. Then introduce at this point a sterile platinum spud, with which tubes of culture media are inoculated. The platinum loop may be used where an incision has been made into the organ with a sterile knife.

A bacteriological capillary pipette is a good instrument for taking up blood from right heart or blood vessels. When great precaution is necessary to insure sterilization of the surface, as in cultures of an excised gland or organ, the piece of tissue may be dropped into 5% formalin solution for a few minutes, washed in sterile salt solution, next placed in a sterile Petri dish and the material obtained from the center; or it may be dropped for a few seconds into boiling water. Before performing a necropsy on experimental animals it is well to dip the dead animal into 3% trikresol solution.

In autopsying small laboratory animals they may be tacked down by each foot to a wooden board, the whole being thrown in the furnace after the autopsy. When pans are used these should be put in the autoclave. It is advisable to use rubber gloves when autopsying animals infected with virulent organisms. To avoid conjunctival infection it is well to wear goggles.

### NATURAL INFECTIONS OF LABORATORY ANIMALS

In human autopsies we appreciate the importance of great experience in recognizing the pathological lesions caused by the various diseases affecting man. In the study of the lesions in experimental animals experience is equally important. It is essential to be familiar with the appearance of the normal organs of such animals, and with the changes in the tissues and body fluids caused by not only the infection with which the animal has been inoculated, but also those diseases which may occur spontaneously in the animal. In view of the rigid controls demanded in other fields of research, it is remarkable that relatively so little attention has been given to this matter.

These notes are mainly from McIntosh's article in "System of Bacteriology" (Medical Research Council), and from that of Juanita Thompson (1936) on spontaneous virus diseases in common laboratory animals. It is very hard to recognize those virus infections of animals which fail to show either clinical or postmortem manifestations.

Mice. "Mouse typhoid," due usually to some species of Salmonella, especially S. aertrycke, is an important cause of fatal epizootics in laboratory-bred animals. A disease resembling lymphatic leukaemia is rarely encountered. Tumors—especially adenocarcinoma of the breast—are not uncommon. Sarcosporidiosis, ring worm, and helminthic infections (larval and adult) also occur.

Virus diseases. Marschal's infectious ectromelia is characterized by cutaneous infections progressing to gangrene, or by abdominal lesions. Cytoplasmic inclusions may be present. Diseases of the salivary glands and lymphocytic choriomeningitis due to viruses not found in man have been reported. Theiler's disease, which causes a flaccid paralysis of the hind legs, occurs spontaneously.

Guinea pigs.—Spontaneous diseases of these animals are comparatively rare. Both glandular and septicaemic forms of pseudotuberculosis rodentium may affect these animals. Respiratory and intestinal affections of bacterial origin may occur when overcrowding or unhygienic environment exists. Coccidiosis and tumors have been reported occasionally.

Virus diseases.—A virus infection of the salivary glands, and one causing "guinea pig paralysis" may occur.

Rabbits.—Rabbits suffer spontaneous infections with pseudotuberculosis, usually due to Pasteurclia pseudotuberculosis-rodentium. Other species, notably P. cuniculicida, cause "snuffles," which may give rise to a fatal pneumonia, or to sinusitis, septicaemia or meningitis. Species of Salmonella, particularly S. aertrycke, may cause outbreaks of enteritis. Diarrhoea may also be due to coccidiosis, a very common infection in rabbits. Infections occur with Trypanosoma lewisi, and with Treponema cuniculi, which involves the genitalia and may be confused with T. pallidum of man.

A spontaneous encephalitis, probably due to a protozoan, has been reported in Europe and America. Fungus infections are common. Mites may cause skin lesions about the ears. The commonest helminthic infection is with the larval stage of *Taenia pisiformis* of the dog. The cysticerci are found in the omentum and liver of infected rabbits. Tumors are rare.

Virus III, described by Rivers, causes an acute orchitis.

Rats.—If properly cared for, rats are relatively free from spontaneous diseases, except mange which especially involves the ears and tail. Respiratory and intestinal infections occur. The liver may show the larval stage of the cat tapeworm.

Virus infections may involve the salivary glands, causing intranuclear and cytoplasmic changes in the cells of the ducts and parenchymatous tissues. Hindle has described intranuclear inclusions in the cells of apparently normal London rats.

Monkeys.—Once acclimatized, monkeys are comparatively healthy. Intestinal tuberculosis can be avoided by feeding pasteurized milk. They may contract pseudo-tuberculosis from associated rodents.

The virus of herpes may be indigenous in monkeys. Virus B may spontaneously produce various clinical manifestations. There seems to be an immunological relationship between this virus and those of herpes and pseudorabies. Lymphocytic choriomeningitis also may be harbored by monkeys. Intranuclear inclusions have been observed in the nasal mucosa, trachea, lungs and bile ducts of normal monkeys.

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION

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Disease	Material	Animal	Method of inoculation	Period of incubation	Pathological lesions	Sepsis	Remarks
Anthrax.	Material from the pustule,	Mouse, guinea S.c. pig.	S.c.	I to 2 days.	Gelatinous exudate at site of inoculation.	Extreme, blood al- most black.	Bacilli particularly numerous in the spleen.
Botulism,	Filtered extract of suspected food.	Mouse, guinea S.c. pig.	S.c.	I to 4 days.	Death due to cardiac or respiratory failure. May show oculomotor paralyses and mydriasis.	0	Inoculate a control animal together with antitoxin.
Brucellosis.	Blood, urine or material from local focus.	Guinea pig (male).	S.c.	I to 3 months.	Caseous nodules in spleen, liver, lymph glands. Of- ten caseous epididymitis.	+	Animals not very susceptible to bovine type. Agglutins may be demonstrable.
Cholera.	Culture.	Guinea pig.	I.p.	I to 2 days.	Perttonitis.	0	To identify vibrio inocu- late a control animal, together with anticholera serum. Examine the per- loneal exudate for bac- teriolysis at 30 minute intervals. (Pfeiffer.)
Diphtheria.	Broth culture or filtrare (2 cc., 48 hours).	Guinea pig.	S.c.	2 to 4 days.	Local oedematous swelling with enlarged regional glands. Pleural and peritoneal haemorrhagic effusions. Bularged haemorrhagic adrenals.	0	Inoculate a control animal together with 500 units of antitoxin. Virulence may also be tested intradermally (p. 87).
Friedländer pneumonia.	Sputum,	Mouse.	S.c. or I.p.	I to 2 days.	Peritoneal exudate very sticky.	+	Rabbits and guinea pigs are refractory to sub- cutaneous injection.
Gas gangrene.	Material from wound; or irroth culture.	Gumea pig.	Ė	ı to 2 days.	Pathogenicity varies. Infammation and necrosis of tissue with gas formation.	-11	Differentiate species by control animals receiving also specific antitoxic sera.
Glanders.	Material from le- sion,	Male guinea pig.	I.p. or S.c. if contam- inated.	2 to 3 days.	Generalized granulomata. Barly orchitis from which organisms can be isolated. (Straus reaction.)	Rare.	Consider Actinobacillus pseudomallei.

Lobar pneumonia.	Sputum. Material   Mouse. from lung punc- ture.	Mouse.	I.p. or S.c.   1 to 5 days.	I to 5 days.	General infection with per- itonitis.	+	Isolation from blood possible but cultures are preferable. For type determinations, etc. see p. 38.
Plague.	Material from bubo, sputum, blood.	Mouse, rat, guinea pig.	Cut., S.c. or 2 to 5 days.	2 to 5 days.	Haemorrhagic lymphadenitis. Caseous areas in glands, spleen, liver, occasionally other organs.	+	The ability to infect through the unbroken skin is of some value in diagnosis.
Tetanus.	Material from wound; preferably filtrate from broth culture.	Rat, mouse, guinea pig.	S.c.	I to 5 days.	Tetanus. Spasticity begins in muscles nearest point of inoculation.	o	Inoculate a control animal and give a prophylactic dose of antiteranic serum. Rats show typical "seal gait."
Tuberculosis.	Sputum, urine, spinal fluid, exudates, etc.	Guinea pig.	S.c.	4 to 8 weeks.	Local caseous lesions with involvement of regional, and later other lymph glands. Tubercles in spleen, liver and other organs.	0	If material is badly contaminated, treat with alkali, wash and neutralize before injection.
Tularaemia.	Material from pri- mary lesion, lymph nodes or blood.	Mouse, guinea pig, rabbit.	S.c., I.p.	2 to 7 days.	Haemorrhagic lymphadenitis. Caseous areas in glands, spleen, liver, and occasionally in other organs.	+	Cutaneous inoculation will infect. Agglutinins can be demonstrated if animal lives longer than 10 days.
Relapsing fever.	Blood.	Mouse, rat.	I.p.	2 to 5 days.	Organisms appear in blood after 1 to 2 days and persist for 2 to 4 days.	+ '	Relapses may occur in mice. Infection never fatal.
Weil's disease.	Blood, urine.	Guinea pig.	I.p.	7 to 12 days.	Jaundice. Haemorrhages in lungs, serous cavities, muscles, etc.	+	Leptospiras can be demon- strated in blood and tissues.
Rat-bite fever.	Local lesions, or regional glands, blood.	Mouse, rat, guinea pig.	I.p.	5 to 14 days.	Examine blood. In guinea pigs pyrexia and enlarged lymph glands.	+	Rats and mice develop a sepsis but do not die.
Epidemic typhus.	Blood 1 cc.	Guinea pig I.p. nearly grown male.	I.p.	After 5 to 12 days, fever lasting 5 to 9 days.	No gross scrotal or testicu- lar lesions. Animal re- covers.	+	Blood and brain infective during fever. After re- covery animal is immune.
Endemic typhus. Blood 1 cc.	Blood I cc.	Guinea pig nearly grown (male).	Lp.	After 5 to 12 days, fever lasting 1 to 9 days.	Marked swelling and ery- thema of scrotal skin, Animal recovers,	+	Ѕате.

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION (Continued)

	TOUTO	TIME TO CITE	ייית פטטווט	זעני זע פאפטאט	Control of Intections Districted Interest Incomment	(	
Disease	Material	Animal	Method of inoculation	Period of incubation	Pathological lesions	Sepsis	Remarks
Rocky Mountain Blood 1 cc. spotted fever.	*	Guinea pig I.r nearly grown (male).	L.p.	After 2 to 10 days, fever lasting 5 to 15 days.	In severe western type only, scrotal skin shows thrombosis and necrotic oedema leading to gargree. Mortality 95 %. In eastern type no scrotal lesions. Mortality 40 to 50 %.	+	Зате.
Coccidioidal granuloma.	Pus.	Guinea pig.	S.c.	4 weeks.	Typical lesions showing endogenous sporulation.	0	Useful in differentiation from blastomycosis.
Trypanosom- iasis.	Blood, gland juice.	Rat, guinea pig.	I.p.		Examine blood at intervals after about two weeks.	+	
Leishmaniasis.	Blood or material aspirated from glands, spleen, mar- row, etc.	Chinese hamster, mouse, rat, dog.	I.p., large doses.	Long and variable.	Generalized lesions similar to human disease.	0	Inoculation usually unsuccessful. Not a useful diagnostic procedure at present. Has now been produced experimentally by insect vectors.
Dengue.	Blood in first 3 days.	Monkeys.	S.c.	5 days.	Typical dengue.	+	,
Epidemic encephalitis (St. Louis type).	Brain emulsion.	Monkeys, mouse (passage virus).	I.c. I.n.	8 to 14 days. 5 to 7 days.	Encephalitis.		Relation to virus of Austra- lian X disease (now iost) and Japanese Type Bencephalitis not krown. Serum from cases of Economo type does not protect from St. Louis type.
Herpes febrilis.	Vesicular fluid, sa- liva, blood, spinal fluid.	Rabbit, mouse	Corneal I.d.	4 to 7 days.	Keratitis. Sometimes en- cephalitis. Papular eruptions.		Inclusion bodies in corneel epithelium.
Foct-and-mouth disease.	Milk, saliva, ex- creta; fluid of vesi- cles, blood.	Guinea pig.	I.p., S.c. Cut. I.n.	4 days.	Typical lesions.	-1-	Threeimmunological types, O, A and C.
Influenza.	Filtrate of nasal secretion.	nasal Ferret, mouse. I.n.	L.n.		Mice often develop pneumonia due to virus alone or secondary invaders.	0	Ferret used for isolation.

Louping ill.	Tissues of brain Mouse, and cord.	Mouse, rat, I.c. sheep.	I.c. I.n.		Encephalitis, especially of Purkinje cells of cere- bellum.		Inclusion bodies in nerve cells.
Lymphocytic chorio-menin- gitis.	Brain, spina! fluid, blood.	Guinea pig, monkey, mouse.	S.c., I.p., I.c., I.n.	2 to 9 days.	Typical choriomeningitis. Bronchopneumonia. Sometimes necrotic areas in other organs.	+	Chronic lesions may persist after recovery, especially in kidney. Animal becomes a carrier.
Lymphogranu- loma inguinale.	Pus from bubo.	Mouse, guinea I.c. pig.	I.c.		Encephalitis.		Brain emulsion can be used to prepare Frei antigen.
Poliomyelitis.	Brain or cord emulsion.	Monkey, rabbit?	I.c., S.c., I.p., I.n.		Typical disease	Transient in ani- mals.	Virus not present in human C.S.F. Nota diag- nostic procedure.
Psittacosis.	Filtered sputum, blood.	Mouse, guinea I.p. pig, parrot.	I.p.	7 to 10 days.	Focal necroses in liver and spleen. Psittacosis bodies in reticuloendothelial cells.	+	Liver lesions diagnostic.
Rabies.	Brain, saliva.	Guinea pig, rabbit.	Subdural.	Within 3 weeks.	Negri bodies in hippo- campus, fissure of Ro- lando, cerebellum.	0	Useful if suspected dog is decomposed.
Rift Valley fever. Blood, tissues	Blood, tissues	Mouse,	I.p., S.c., I.n. Cut.	2 to 5 days.	Pocal necroses in liver with intranuclear inclusion bodies.	+	Liver lesions diagnostic.
Variola.	Material from skin Rabbit. lesions.	Rabbit.	I.d. Corneal.	5 days. 2 to 3 days.	Local reaction. Keratitis.		McKinnon's test. Paul's test.
Yellow fever.	Blood (first 3 days) liver, spleen.	(a) Mouse, guinea pig. (b) Monkey.	I.c. or I.p. after I.c. injection of starch. I.p.	5 to 10 days.	(a) Encephalitis (b) Typical lesions in viscera.	+	Protection tests used to detect presence of anti- bodies in epidemiological studies. Characteristic lesions in the liver with intranu- clear inclusions.
Trichinosis.	Suspected food.	Mouse, rat.	Feeding.	2 weeks.	Examine diaphragm for encysted larvae.	Transient.	Not as much used now as other tests,

Cut. = cutaneous.
I.c. = intracerebral.
I.d. = intradermal.
I.n. = intransal.
I.p. = intraperitoneal.
S.c. = subcutaneous.

## CHAPTER II

# STUDY AND IDENTIFICATION OF BACTERIA—COCCI. KEY AND NOTES

Streptococcus Forms.—Cells in short or long chains, never in packets.

Streptococci are divided into three main groups by the appearance of the colonies on blood agar. To these groups we have added a fourth to include a number of species which require complete or partial anaerobiosis for their growth. Various investigators have attempted to differentiate species within these groups by their ability to ferment different carbohydrates (especially lactose, mannite and salicin) and by agglutination and agglutinin absorption tests, but the results obtained by these methods do not always correspond. The following varieties have been differentiated by Holman and others, although the differences are not sufficiently sharp in all cases to warrant considering them as separate species.

- I. Parasitic or semiparasitic.
  - A. Haemolytic group.
    - (a) Produce wide zone of haemolysis around colonies on blood agar.

Type  $\beta$  (beta) of Smith and Brown. In this group belong the following types:

- 1. Streptococcus pyogenes (S. haemolyticus).
  - a. S. scarlatinae.
  - b. S. erysipelatis.
  - c. S. puerperalis.
- 2. Streptococcus mixtos (S. infrequens).
- 3. Streptococcus equi.
- 4. Streptococcus cuniculi.
- 5. Streptococcus felini.
- (b) Produce narrow zone of haemolysis around colonies on blood agar.
  - 6. Streptococcus stenos.
- B. Viridans group. No haemolysis. Colonies green surrounded by a greenish zone due to production of methaemoglobin.

Type  $\alpha$  (alpha) of Smith and Brown.

- 1. Streptococcus mitior (S. viridans, S. mitis).
- 2. Streptococcus salivarius.
- 3. Streptococcus faecalis.
- 4. Streptococcus mastitidis.
- 5. Streptococcus equinus.
- 6. Streptococcus bovis.
- 7. Streptococcus ignavus.
- C. Non-haemolytic group. Produce no change on blood agar. Type  $\gamma$  (gamma) of Smith and Brown.
  - 1. Streptococcus anhaemolyticus.
  - 2. Streptococcus saprophyticus.

Note.—An alpha prime type is also recognized, intermediate between the alpha and beta types. The colonies are surrounded by an indistinct zone of haemolysis, which, under low magnification, shows intact red cells.

- D. Anacrobic group. Many strains produce foetid gas in cultures. Found in puerperal sepsis, lung abscess, gangrenous infections of the mucous membranes. Often associated with the spirochaetes and fusiform bacilli of Vincent.
  - 1. S. putridus.
  - 2. S. foetidus.
  - 3. S. anaerobius.
- II. Saprophytes occurring chiefly in dairy products and found principally in sour milk and in the ripening process of several varieties of cheese. In this group belong the lactic acid bacteria including S. cremoris, S. thermophilus, S. lactis, S. kefir, and several other non-pathogenic species.

**Diplococcus Forms.**—Cells usually in pairs. Grow poorly on artificial media. Oval to lancet-shaped forms, less frequently in chains. Colonies greenish on blood agar. No haemolysis. Bile soluble.

(i) Diplococcus pneumoniae (Pneumococcus). Three principal types recognized. (See text.)

Staphylococcus Forms.—Cells as a rule in irregular groups, rarely in packets. Pigment white or orange, or less commonly lemon-yellow.

- I. Orange pigment. Lactose fermented. Gelatin liquefied.
  - (1) Staphylococcus aureus.
- II Lemon-yellow pigment.
  - (2) Staphylococcus citreus.
- III. White growth on solid media.
  - (a) Ferment lactose, sucrose, liquefy gelatin.
    - (3) Staphylococcus epidermidis.
  - (b) Ferment lactose, sucrose, mannitol.
    - · (4) Staphylococcus albus.
  - (c) Ferment lactose, sucrose, mannitol, raffinose.
    - (5) Staphylococcus pharyngis.

Tetragena Forms.—Cells in tetrads, surrounded by a capsule. Ferment glucose and lactose.

(1) Gaffkya tetragena. (M. tetragenus).

Sarcina Forms.—Division occurs in three planes, producing regular packets. Growth on agar abundant, usually with the formation of yellow or orange pigment.

- I. Non-motile forms.
  - A. Yellow pigment formed.
    - (a) Gelatin not liquefied.
      - (1) Sarcina ventriculi (stomach contents of man and animals).
      - (2) Sarcina conjunctivae (conjunctiva).
    - (b) Gelatin liquefied.
      - (3) Sarcina flava (air).
    - (4) Sarcina lutea (air, soil, water).
  - B. Orange pigment formed.
    - (5) Sarcina lactea (fresh milk).
    - (6) Sarcina aurantiaca (air, water)

- C. No pigment formed. Gelatin not liquefied.
  - (7) Sarcina hamaguchiae (soy bean mash).
- II. Motile forms.
  - A. Yellow pigment formed. Gelatin not liquefied.
    - (8) Sarcina citrea (air).
    - (q) Sarcina ureae (urine).

Micrococcus Forms.—Cells in plates or irregular masses (never in chains or packets). Facultative parasites. Found in oral cavity and urine. Aerobic and anaerobic species Non-pathogenic for man.

Gram Negative Cocci. Neisseria.—Cells normally in pairs.

- A. Grow only at 37°C. on special culture media containing blood, blood serum, starch or on plain agar with vitamin.
  - (1) Neisseria gonorrhoeae (Gonococcus).
  - (2) Neisseria intracellularis (Meningococcus). Four distinct serological types.
- B. Grow well at 22°C. on ordinary culture media.
  - (a) Non-chromogenic.
    - (3) Neisseria catarrhalis.
    - (4) Neisseria sicca.
  - (b) Chromogenic.
    - (5) Neisseria perflava.
    - (6) Neisseria flava.
    - (7) Neisseria subflava.

## STREPTOCOCCUS FORMS

Occurrence.—Streptococci are widely distributed in nature and are the cause of a great variety of infections, both in man and animals. They are frequently found in the normal mouth, nose and intestinal tract, and also in water, milk and dust. On this account the occurrence of streptococci of any type in cultures, particularly those from the respiratory tract, must be interpreted with great care.

Morphology.—The streptococcus is a round, Gram-positive coccus occurring typically in chains, especially in fluid media. It often occurs as a diplococcus in blood. Streptococci have been differentiated into short and long chain varieties; the virulent types usually occur in chains of from ten to twenty cocci, whereas the normal inhabitants of the nose, mouth and intestinal tract tend to be in short or, sometimes, very long chains. This grouping is very rough, however, since chain formation depends to a considerable extent upon cultural and other conditions. There is an extraordinary difference in size in various strains, and many of them, especially in old cultures, show bizarre, elongated, or club-shaped forms. Occasionally capsules can be demonstrated.

Cultural characteristics.—Most streptococci are aerobic. Some, however, are strictly anaerobic and others require a partial oxygen tension. They grow best at 37°C, on media enriched with blood or serum. On agar plates the surface colonies are small, greyish, and round, while deep colonies are lenticular in shape. The appearance of the colonies on blood agar (free from carbohydrates) is quite characteristic, and forms the basis for the differentiation of the following groups (Schottmüller, Brown).

#### HAEMOLYTIC AND VIRIDANS TYPES

- 1. Haemolytic group (beta type). Colonies surrounded by a clear zone of haemolysis.
- 2. Viridans group (alpha type). Colonies green with a surrounding greenish discoloration of the blood agar, due to the formation of H<sub>2</sub>O<sub>2</sub> and methaemoglobin.
- 3. Non-haemolytic group (gamma type). Colonies greyish. No change in the surrounding medium.

In broth (preferably pH 7.4 to 7.6) the short-chain streptococci produce a diffuse or granular turbidity, whereas the long-chain types tend to form a floculent growth which adheres to the side of the tube, and after three or four days falls to the bottom. This tendency to flocculation in streptococcus cultures causes difficulty in evaluating the results of agglutination tests. Frequent transfers on special media may be necessary to minimize this spontaneous clumping but this may result in a change in their characteristics. The addition of glucose to the broth facilitates growth if calcium carbonate (1%) is added to absorb the acid produced by fermentation of the glucose. Some freshly isolated strains will not grow unless the medium is enriched with serum or blood. Milk is acidified and usually coagulated.

Many of the carbohydrates are fermented, generally without gas formation. In testing these reactions it is necessary to add the various sugars to media such as Hiss' serum water, in which the streptococci grow luxuriantly. Species differentiation of streptococci has been attempted by their action on lactose, mannitol and salicin, but the biochemical activities of the different species are somewhat variable, and the results obtained do not correspond with those of serological tests. Streptococci do not ferment inulin, though a few non-haemolytic strains and a type designated as S. mucosus, resembling the Type III pneumococcus, are exceptions to this rule.

Differentiation from the pneumococcus.—The most important criterion in differentiating streptococci from pneumococci is the solubility of the pneumococcus in bile. To carry out this test, add to 2 cc. of a 24 hour broth culture of the organism about 0.1 cc. of bile (or a 10% solution of sodium taurocholate). The mixture should remain in the incubator for one half hour. Streptococcus cultures remain unchanged, whereas the turbidity of the pneumococcus culture clears up. The solution can also be observed in a hanging drop preparation.

Virulence and viability.—The virulence of streptococci is exceedingly variable. Cultures recently isolated from human infections are not very virulent for animals, and usually cause only abscesses or other localized lesions. By successive passages through rabbits (or mice), however, the virulence of a strain for this species can be greatly increased, so that it causes a generalized infection fatal within two or three days. Cultures on ordinary media usually die out within two weeks and lose their virulence rapidly. This may be restored by successive passages through rabbits, preferably by intravenous injections. Both viability and virulence are best maintained on chocolate agar or other albuminous media. Cultures must be kept in the ice box, and should be subcultured every two to four weeks.

Variation.—Todd has described dissociative changes in haemolytic streptococci after prolonged cultivation on artificial media. When freshly isolated the colonies are "matt-surfaced," later they become smooth and glossy. This change is accompanied by a permanent loss of virulence, although the production of the haemolysin is not affected. Recently Loewenthal has reported that the matt-surfaced colonies are changed by mouse passage into a mucoid form as the virulence of the streptococcus for mice is enhanced.

Toxins.—Haemolytic streptococci produce soluble toxins in their growth in vivo and in vitro. The presence of an haemolysin may be demonstrated by adding 1 cc. of an 18 hour serum-broth culture to an equal volume of a 5% suspension of red cells and incubating for an hour and a half. The haemolysin is inactivated by heating at 55°C, and by oxidation. Its formation does not parallel the virulence of the strain for animals. Streptococci of the viridans group also form an haemolysin, which, however, is liberated only after autolysis. Streptococci also form a thermolabile leukocidin which inhibits phagocytosis. Tillet and Garner (1933) demonstrated the formation of a librinolysin which liquefies the clot from normal human (but not rabbit) blood. The clot from the blood of patients convalescent from a streptococcal infection, however, is not liquefied, and the serum from such a patient inhibits fibrinolysis of a normal clot.

The Dicks (1924) demonstrated conclusively the presence of a soluble exotoxin in culture filtrates of haemolytic streptococci from patients with scarlet fever. This toxin is relatively resistant to heat (even 100°C. for brief periods) and other injurious agencies. It is neutralized by the serum of convalescents and stimulates the production of specific antitoxin in animals. (Its activities are described below.) Similar toxins are formed by streptococci from other sources, but the species-specificity of these different toxins is still disputed.

Serological differentiation.—Classification of the streptococci has been attempted by means of cross-agglutination, agglutinin absorption and protection tests with divergent results. No definite groups have been demonstrated among the non-haemolyzing strains. Among the haemolytic streptococci, however, serological relationships have been reported in streptococci isolated from cases of scarlet fever (Bliss, Dochez, the Dicks), from erysipelas (Birkhaug) and from puerperal sepsis (Lash and Kaplan). Such groups are not clear cut, however, and other investigators question their validity. The explanation of this confusion may be found in the complex antigenic structure of the streptococci. Lancefield has separated three antigenic constituents: (1) a nucleoprotein common to the genus and related to that of the pneumococcus and the staphylococcus; (2) a carbohydrate haptene specific for the haemolytic group; and (3) a type-specific haptene which is probably protein in nature. Furthermore, the antigenic properties of a single strain may vary. The type-specific haptene is lost if a strain is dissociated to the glossy type.

Susceptibility to drugs.—Sulfanilamide (para-amino-benzene-sulphonamide) or prontosil exerts a bacteriostatic effect on the growth of haemolytic streptococci in vitro. It also exerts a protective and curative action in mice infected with virulent strains of haemolytic streptococci. Long and Bliss (1937) believe that this effect is due to some change in the streptococci which make them susceptible to phagocytosis.

#### HAEMOLYTIC STREPTOCOCCUS INFECTIONS

The haemolytic streptococci cause inflammations which are diffuse and phlegmonous in character with tissue necrosis rather than pus formation, whereas the staphylococci produce circumscribed, purulent lesions. The ordinary follicular tonsillitis is frequently caused by streptococci of the beta type, and severe epidemics of sore throat have been spread by contaminated milk from cows whose udders were infected by human carriers of these streptococci. (The type of streptococcus, *S. mastitidis*, which causes the chronic mastitis of cows is alpha or alpha prime.) They are frequently secondary invaders in diphtheria, tuberculosis, small-pox and even in typhoid fever, and are the most common cause of the severe broncho-pneumonias which may follow the acute infectious diseases, particularly measles and influenza.

Haemolytic streptococci are frequently the cause of wound infections, of otitis media, mastoiditis and sinus thrombosis, and of puerperal infections. A rapidly fatal septicaemia with or without a meningitis may result from any of these conditions. Streptococci cause various diseases peculiar to animals, such as "strangles" in the horse, and contagious mammitis in cows.

## Scarlet Fever

Klein in 1886 isolated from the throats of scarlet fever cases haemolytic streptococci which he called *S. scarlatinae*. This observation has been repeatedly confirmed, and they have also been grown from various suppurating lesions, and from the blood in fatal cases. Evidence that this organism is the cause of scarlet fever has been advanced in recent years by Dochez and the Dicks.

In selected volunteers the latter succeeded in producing scarlet fever experimentally by swabbing the throats with haemolytic streptococci obtained from a case of the disease. This observation was confirmed by Nicolle in 1926. The Dicks showed also that the S. scarlatinae produces an exotoxin which can be demonstrated in Berkefeld filtrates of broth cultures, and which, when injected intradermally into susceptible individuals, produces a characteristic skin reaction. The toxin is standardized by determining the minimum amount which, when injected intracutaneously, will induce a certain standard reaction.

The Dick test.—This test is used to determine susceptibility to scarlet fever and is carried out in the same manner as the Schick test for diphtheria. Over 80% of adults give a negative reaction to the toxin and have, therefore, a natural antitoxin (and perhaps other antibodies) in the blood. The test is usually positive during the early stages of scarlet fever and gradually disappears during convalescence.

A saline dilution of the standard toxin with a potency of one skin-test dose in o.r cc. is used. This is injected intradermally in the flexor surface of the forearm. Positive reactions appear in from four to twelve hours and are read in twenty-four hours. At the height of the positive reaction there is a circumscribed area of redness and infiltration varying from one to three or four centimeters in diameter according to the suscepti-

bility of the individual. A reaction which has entirely faded in twenty-four hours is negative. Pseudoreactions are rare and controls are not necessary.

Immunization.—An active immunity can be produced by four or five subcutaneous injections of increasing doses of the toxin at weekly intervals. The Dicks recommend the following dosage; (1) 500 S.T.D.; (2) 2500 S.T.D.; (3) 20,000 S.T.D.; (4) 40,000 S.T.D. Three weeks after the last injection the presence of antitoxin is established by another Dick test.

The Schultz-Charlton reaction.—Antiscarlatinal serum or convalescent serum injected intradermally in an erythematous area will cause a definite and permanent blanching of the surrounding scarlatinal rash within five or six hours. This phenomenon is due to a local neutralization of the toxin. It occurs only in the scarlet fever exanthem and is, therefore, a useful diagnostic test in doubtful cases.

Serum treatment.—By immunizing horses with toxic filtrates of S. scarlatinac or with living cultures in agar deposits injected subcutaneously, as suggested by Dochez, an immune serum can be obtained which is used for prophylaxis and treatment. This is standardized by its ability to neutralize a given amount of toxin. One unit is the smallest amount which will neutralize one hundred skin-test doses of a standard scarlatinal streptococcus toxin. A Dick-positive individual may be rendered Dick-negative within 24 to 48 hours by a prophylactic dose, but the protection conferred lasts only for from two to four weeks. Moser, Dochez, and later the Dicks, found that immune serum was effective, when given early, in causing a disappearance of the scarlatinal rash and in relieving symptoms. The dosage recommended is from 2000 to 10,000 units, or more in severe cases. Convalescent serum obtained from the fourth to the seventh week has also been used intramuscularly in doses of 50 to 100 cc. Immune serum must be given early to be effective. It appears to be of no use after complications have developed.

## Erysipelas

Birkhaug claims that by means of agglutination and agglutinin absorption tests he has demonstrated that over 90% of haemolytic streptococci obtained from cases of erysipelas fall into a definite group which is serologically distinct from those isolated from cases of scarlet fever, and also from those from common septic infections. A serum prepared from the erysipelas strains protected rabbits from infection with similar strains, but did not confer immunity to streptococci from other sources. Intracutaneous injections of these erysipelas strains into normal rabbits produced a condition resembling human ervsipelas, and with a toxin obtained from the organisms a skin reaction akin to the Dick test could be demonstrated. He further reports good results in treatment with an antiserum prepared with erysipelas streptococci. When administered early the injection was usually followed by a critical drop in temperature, an amelioration of the general condition and a disappearance of the eruption. Others have confirmed these therapeutic results. However, Williams, Wadsworth and others have observed cross agglutinations between these streptococci and those from cases of scarlet fever, and McCann and others have found that scarlet fever antitoxin is as effective as erysipelas antitoxin in the treatment of erysipelas.

## NON-HAEMOLYTIC STREPTOCOCCUS INFECTIONS

Non-haemolytic and viridans streptococci are frequently the cause of infections of the tonsils, sinuses, middle ear, and teeth, and also of the gall

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bladder and appendix. The lesions produced are of a more chronic type than those due to haemolytic streptococci, and are frequently overlooked. The great importance of these infections is the part which they play in the various focal infections (see Chap. XL). Other organisms may also be concerned, but streptococci, particularly the non-haemolyzing types, are by far the most important etiological agents.

#### Subacute Bacterial Endocarditis

This is the most serious disease due to the non-haemolytic streptococcus. The portal of entry of the organism is presumably some chronic focus of infection, probably most often infected teeth or tonsils. In some cases the endocarditis has started acutely immediately after a tonsillectomy or a tooth extraction. The organisms lodge in the heart valves, but usually only when these have been damaged previously by a rheumatic infection. Here they live and multiply in vegetations in which they appear to be protected from the leukocytes and antibacterial substances in the blood. Embolic phenomena are frequent; arthritis may occur, although without suppuration such as may occur in other forms of sepsis. The disease may last for months or even several years, and remissions are not infrequent, but the outcome is almost invariably fatal. The organisms are present in the blood stream and may be grown from the blood by the ordinary technique. Growth is usually slow, and colonies appear in the plates as a rule in two or three days, occasionally only after five or six days. During the remissions the bacteria may disappear from the blood, and repeated blood cultures may be necessary to demonstrate them. In the later stages they may be present in the blood in enormous numbers, sometimes many thousand per cc. A positive blood culture, however, is not necessarily serious if the heart is normal. Transient invasion of the blood stream has been demonstrated repeatedly and probably occurs more often than is now recognized. Even in the presence of chronic valvular disease, growth of a few streptococci in blood culture does not justify a definite diagnosis of bacterial endocarditis except in the presence of embolic phenomena or other clinical manifestations of the disease. Agglutinating and complement-fixing antibodies are developed in the body in some cases, but not with sufficient regularity to be of diagnostic value. Other organisms (Pfeisfer bacillus, pneumococcus, gonococcus, etc.) may cause a similar clinical picture in about 5% of the cases. This condition is easily differentiated from sepsis and endocarditis due to haemolytic streptococci (occasionally staphylococci and pneumococci) in which the clinical picture is that of a fulminating, rapidly fatal infection. The origin of the infection (infected wounds, mastoiditis, puerperal sepsis, etc.) is usually evident. Blood cultures determine the type of organism concerned.

#### Arthritis

There is much indirect evidence that streptococci are the etiological agents both in acute rheumatic fever with which chorea and endocarditis are associated, and also in chronic infectious (rheumatoid, atrophic) arthritis. There is controversy, however, both as to the type of streptococcus concerned and also as to the mechanism by which the arthritis is produced. In both conditions streptococci (most frequently non-haemolytic in type) have been cultivated from the blood and joint fluids by some investigators (Poynton and Paine. Rosenow, Small, Birkhaug, Cecil, and others),

whereas many others have obtained negative results, even with the same methods of isolation. Chronic foci of infection containing streptococci are frequently demonstrable, and their removal is often followed by temporary aggravation and subsequent relief of the arthritis. Cultures of streptococci (made in deep tubes of glucose serum or brain agar) can frequently be obtained from the enlarged lymph glands which drain the diseased joints. Furthermore, these patients often give positive allergic skin reactions to streptococci from local foci or to some stock streptococcal strains, and their serum often shows a high agglutinin titer to them. An exacerbation of acute rheumatic fever is often preceded by an acute (haemolytic) streptococcal throat infection, and the serum often shows an increase in anti-strepto-haemolysins (though usually not in agglutinins). It is probable that both conditions are associated in some way with streptococcal infection; either as the result of a direct metastatic infection, or of the action of some circulating toxin, or of a local allergic reaction in the affected joints.

Vaccines made from strains of streptococci isolated from foci of infection, or from regional lymph glands, or from stock strains which are actively agglutinated by the patient's serum, or to which the patient gives a positive allergic skin reaction have been used extensively in the treatment of chronic infectious arthritis and seem to be definitely beneficial in many cases. It is uncertain whether such results are attributable to a specific immunization, to a desensitization of the tissues, or to a non-specific foreign protein reaction.

Glomerular Nephritis.—Indirect evidence of a similar character indicates that betahaemolytic streptococci are probably the cause of many cases of acute glomerular nephritis. Many cases are associated with, or quickly follow, acute infections with this organism, particularly scarlet fever and acute follicular tonsillitis. Such cases often give a positive skin reaction to toxic filtrates of haemolytic streptococci and show a high titer of anti-strepto-haemolysins in the blood (Longcope, 1929, 1936). Winken werder et al. (1935) found that in the acute cases which have followed an acute infection. recovery was the rule, but when associated with a chronic or recurring infection the nephritis was progressive.

Pathogenic anaerobic streptococci have been isolated from a number of conditions, particularly lung abscesses, often in association with the spirochaetes and fusiform bacilli of Vincent. They have also been found in the blood in puerperal sepsis (10%), to 40%). Studies made by Colebrook (1930) showed that these strains were serologically heterogeneous, and that some of them produced foetid gas when blood was present in the medium (Schottmüller's S. putridus).

Many varieties of streptococci have been isolated from various sources and named accordingly (e.g., S. salivarius, S. fecalis). This discussion has been limited to a few important types, the identity and etiological relationships of which have been established. Probably no other organism is responsible, directly or indirectly, for such a wide variety of acute and chronic pathological conditions.

#### PNEUMONIA AND OTHER PNEUMOCOCCUS INFECTIONS

Diplococcus pneumoniae (*The Pneumococcus of Fraenkel*) Pasteur and Sternberg, 1880; Fraenkel, 1884.—The pneumococcus is by far the most common cause of lobar pneumonia (over 90%) and may also cause bronchitis, bronchopneumonia, conjunctivitis and corneal ulcers, otitis

media, brain abscess, meningitis, endocarditis, arthritis and other conditions. It is frequently present in the normal mouth.

The following table lists the organisms isolated from 529 cases of lobar pneumonia and their relative frequency, as reported from the Rockefeller Hospital.

Diplococcus pneumoniae (Pneumococcus)	454
Friedländer's bacillus	3
Hemophilus influenzac (Pfeiffer)	6
Streptococcus pyogenes	7
Streptococcus mucosus	r
Staphylococcus aureus	3
Cases of mixed infection with combinations of Staphylococcus aureus,	
Friedländer's bacillus, H. influenzae, Streptococcus pyogenes, and	
Streptococcus viridans	6
Undetermined (most of them occurring before accurate methods for	
determining the etiologic agent had been devised)	49

Morphology.—The pneumococcus in body fluids and in cultures in albuminous media appears as two lanceolate bodies with bases apposed (less frequently oval and rarely

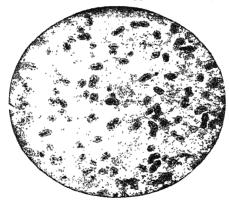


Fig. 2.—Pneumococcus, showing capsule, from pleuritic fluid of infected rabbit, stained by second method of Hiss. (MacNeal.)

round) set in a capsule. In cultures on plain media it does not show a capsule. Short chains occur which are obviously made up of diplococci. Capsules are easily demonstrated by special stains. It is Gram-positive.

Cultural characteristics.—The pneumococcus does not grow below 20°C. On plain agar it grows as a very small dew-drop colony which is slightly greyish by reflected light. It is best cultivated on blood or serum agar (pH 7.6 to 7.8). Deep colonies in blood agar show a zone of olive green discoloration around the colony, whereas colonies on the surface look green by transmitted light, owing to alteration of the blood pigment. There is great variation in the smoothness of the surface of the colonies, depending

upon the size of the capsules. The virulent types which have large capsules produce smooth, often sticky colonies, whereas avirulent pneumococci with small capsules produce small, rough, granular colonies which resemble closely those of the *Streptococcus viridans*.

The pneumococcus ferments all the common sugars, and also inulin, with the production of a considerable amount of acid. It grows readily in meat infusion broth with a pH of 7.6 to 7.8, causing a diffuse clouding of the medium. It grows most profusely in media containing sugars, but is very quickly killed by the acid produced by their fermentation unless this is neutralized by the addition of calcium carbonate. Litmus milk is acidified and usually coagulated.

Viability.—On ordinary media the pneumococcus loses its virulence and quickly dies unless transferred to fresh media. The best media for its preservation are (rabbit's) blood agar or blood broth. In these media virulence is well maintained, and if kept in the ice box the cultures usually remain viable for several weeks. They may be kept alive and virulent for many months by desiccating the spleens of inoculated mice.

Differentiation from streptococcus.—The pneumococcus can be distinguished from the Streptococcus viridans by its fermentation of inulin and especially by its solubility in bile (or 10% solution of bile salts). It must also be distinguished from the pneumobacillus of Friedländer which, although it possesses a capsule like the pneumococcus, is a Gramnegative bacillus.

Types of pneumococcus.—The differentiation of pneumococci into serologically distinct types by Neufeld, and by Cole and his colleagues, is of great practical importance. On the basis of their immunological reactions Cole divided pneumococci into four groups. Groups I, II, and III are usually termed the fixed types; Types I, II, and III. Group IV is serologically heterogeneous and includes all pneumococci which do not belong to the three fixed types.

In cases of pneumonia Type I pneumococci were found in 33%, Type II in 31%, Type III in 12% and group IV in 20%. The mortality was 23, 32, 45 and 10% respectively.

Immune serum prepared with any one of the three fixed types agglutinates, and protects mice from, other strains of pneumococci of the homologous type, but not from those of other types. Avery later studied and designated as Subgroup IIa, IIb, etc., certain strains agglutinating slowly and atypically in Type II serum. More recently 36 other serologically distinct types have been identified by Cooper and others, of which Types IV, V (Avery's IIa), VI, VII, and VIII ("atypical III") seem to be of some importance. Type III corresponds to the *Pneumococcus mucosus*, which was previously differentiated from other types by its moist, sticky growth on albuminous media and by its very large capsule.

Specific polysaccharides.—Avery and others have isolated from the capsular material of each of the fixed types of pneumococci a type-specific soluble substance, which chemically is a complex polysaccharide. The polysaccharide of each type differs chemically from that of every other

type, and the type-specificity depends upon this chemical difference. Although the specific soluble substance is not directly toxic for animals, it endows the pneumococci with their virulence and invasive power. It neutralizes completely in vitro and in vivo the protective power of the homologous immune serum. It is found in the blood serum and body fluids during the active stage of the disease and disappears with recovery.

It is probable that the body defends itself against the pneumococcus by the production of an anti-carbohydrate antibody. The outcome seems to depend primarily upon the relative quantity of specific soluble substance produced by the organism and of the antibody produced by the host. If sufficient antibody is produced, it neutralizes the specific soluble substance in the body fluids and in the capsules of the organisms. They are thus virtually degraded into R forms which are phagocytable. It has been demonstrated by Dochez, Clough and others that the scrum of patients at crisis shows specific protective power, agglutinative and phagocytic activity for the infecting type.

Avery and Dubos extracted from a bacterium isolated from a peat bog an enzyme which exerted a specific lytic action on the capsular polysaccharide of Type III pneumococcus. This enzyme destroyed their capsules and rendered the organisms avirulent and phagocytable. On injection it not only protected mice from virulent Type III strains but exerted some curative action in experimental infection.

Virulence.—The fixed types of pneumococci, when freshly isolated, are very virulent for laboratory animals, especially mice and rabbits. One millionth of r cc. of a broth culture when injected subcutaneously or intraperitoneally into a mouse is often sufficient to kill it in 24 to 48 hours. At autopsy the peritoneal cavity is teeming with organisms, and they can be recovered from the heart's blood. After prolonged cultivation on artificial media the virulence is much diminished but may be restored by animal passage. Many of the Group IV strains, however, have relatively little virulence.

Variation.—Griffith, Dawson and others have changed smooth pneumococci of the fixed type (S form) into rough pneumococci corresponding to Group IV (R form) by growing the fixed types in their homologous immune serum or, less easily, by growing them under unfavorable cultural conditions. These so-called "degraded" strains have little or no capsular substance, are relatively avirulent, and do not react with the type-specific antisera. These investigators have then changed these "degraded" strains into their original type or into other fixed types by injecting them subcutaneously into mice together with a fixed type vaccine or a solution of an alcoholic precipitate of extracts of the specific soluble substance. The "degraded" forms then have changed to the type of pneumococcus from which the vaccine was prepared; or, if not sufficiently "degraded," have reverted to their original type, with a corresponding increase in capsular substance and virulence. Rough and intermediate forms are found in the sputum of convalescents along with the fixed types.

## Laboratory Diagnosis and Type Determinations

Blood cultures.—Pneumococci have been isolated by blood culture in from 30% to 50% of the cases of lobar pneumonia. The presence of a few organisms early in the disease is not necessarily unfavorable. If, however, they are present in large numbers (more than 5 colonies per cc. of blood), the prognosis is bad. A terminal sepsis occurs in nearly all fatal cases.

Isolation from sputum.—The pneumococcus can be isolated from the sputum by washing it in the usual way and plating the material on blood agar. The colonies are easily recognized by their green color. When the sputum is not sufficiently tenacious to be washed, or when speed is desirable, pure cultures can be obtained by inoculating mice intraperitoneally and making cultures from the heart's blood after death, usually in 24 to 48 hours. If no sputum can be obtained, the throat may be swabbed, or lung puncture may be carried out. A fine needle is introduced into the solidified lung, and a few drops of fluid are aspirated. A tube of blood broth is inoculated with the fluid, and a portion is injected intraperitoneally into a mouse. In choosing the area for puncture, one must be sure that the underlying lung is solidified or a pneumothorax may result.

Type determination.—To determine the type of pneumococcus, mix 0.5 cc. of a well grown 18 to 24 hour broth culture with an equal volume of each of the immune sera, I, II, and III, properly diluted, and incubate for one hour at 37°C. Sera I and II are diluted 1 to 20, and the less potent serum III, 1 to 5. Type II serum is used undiluted also, to detect the various subgroups (see table below). Well marked clumps will appear in the tube containing homologous serum. An absence of clumping in all tubes indicates that the pneumococcus belongs to the heterogeneous Group IV. This method gives more constant and dependable results than the rapid methods described below, and should be used to confirm the results of other methods.

Serum I (1:20) 0.5 cc.	Scrum II (undiluted) 0.5 cc.	Serum II (1: 20) 0.5 cc.	Serum III (1:5) 0.5 cc.
++	_	_	
~	++	++	-
	+		Page 1
~			++
_	_		
	(1:20) 0.5 cc. ++	(r: 20) (undiluted) 0.5 cc. 0.5 cc. ++ - ++ +	(1:20) (undiluted) (1:20) 0.5 cc. 0.5 cc. 0.5 cc. ++ ++ ++

Type determination by mouse inoculation.—Inoculate a mouse intraperitoneally, and after 6 to 8 hours remove a small drop of peritoneal exudate with a capillary pipette and examine it microscopically for pneumococci. If these are abundant, kill the mouse and wash out the peritoneal cavity with 2 to 3 cc. of salt solution. Centrifuge these washings a few minutes at low speed to throw down the cells and fibrin. Transfer the supernatant bacterial suspension to a second centrifuge tube and centrifuge at high speed for half an hour. Remove the supernatant fluid and suspend the sediment in sufficient salt solution to make a moderately heavy suspension (about equal to an 18 hour broth culture). This may be used for agglutination tests with the immune sera in place of the broth culture, as described above.

In the case of fixed type infections, the supernatant fluid contains the specific soluble substance. The type of pneumococcus can also be determined from this by the precipitin test (as described below in Avery's method of type determination). The precipitin test (but not the agglutination test) may be used when the peritoneal exudate is contaminated with other organisms.

Microscopic agglutination method.—Sabin has devised a quick method of type determination from exudate aspirated from the peritoneum 3 to 4 hours after inoculation.

Small drops of aspirated material are mixed on a slide with a loopful of x to 10 dilution of Type I, Type II, and Type III serum, respectively. These mixtures are smeared, dried and stained, and observed for microscopic agglutination.

If white mice are not available, the common grey mouse is a satisfactory substitute. However, rapid methods of type determination are available which do not require the use of mice.

Avery's method for type determination ("artificial mouse").—Prepare a medium containing 18 parts of a meat infusion broth, pH 7.6 to 7.8, sterilized without pressure; one part of a sterile 20% solution of dextrose; and one part of defibrinated rabbit blood. This should be tubed in 4 cc. quantities and not reheated. Pneumococci grow very rapidly in this medium and usually outstrip contaminating organisms. Care should be taken to obtain a specimen of sputum from the deep air passages and to avoid mouth secretions. Select a portion the size of a bean and wash even more carefully than for mouse inoculation, passing it 3 or 4 times through sterile salt solution. Grind the sputum in a sterile mortar, add 0.5 to 1.0 cc. of sterile broth, drop by drop, and introduce the suspension directly into the medium. Incubate the tubes for 5 hours at 37°C. Make a smear, stain by Gram, and inoculate a blood agar plate. If pneumococci are numerous, centrifugalize at low speed for two minutes, just enough to throw down the red cells but not the bacteria, and transfer the supernatant fluid to a second tube. There are two methods of proceding from this point.

1. The precipitin test.—To the above supernatant fluid add 1 cc. of sterile bile, and put the tube in the water bath at 37°C. for twenty minutes. If the fluid is not clear at the end of this time, centrifuge. The clear fluid is then used for a precipitin test, as follows:

Set up four tubes as follows:

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Tube 1. 0.5 cc. Serum I (1 to 10) plus 0.5 cc. of fluid.

Tube 2. 0.5 cc. Serum II (undiluted) plus 0.5 cc. of fluid.

Tube 3. 0.5 cc. Serum II (1 to 10) plus 0.5 cc. of fluid.

Tube 4. 0.5 cc. Serum III (1 to 5) plus 0.5 cc. of fluid.
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The test is more sensitive if the fluid is layered over the serum. An immediate specific precipitin reaction occurs in the tube containing the homologous immune serum. Incubation is usually unnecessary. A precipitate in tube 2 and not in tube 3 indicates that the organism belongs to Subgroup II.

2. The agglutination test.—Agglutination tests may be made directly on the fluid after removal of the red cells, as described above, if the culture is practically pure.

Determination of specific soluble substance in sputum (Krumwiede).—Heat the sputum in a water bath to coagulate the albumin, and rub up the coagulum with about 1 cc. of salt solution in a water bath for 5 minutes. The saline, which has taken up the specific soluble substance, is then centrifuged until clear, and 0.2 cc. are layered over 0.2 cc. of each of the three type sera. A cloud at the junction of the fluids ("ring" test) indicates the group. This method is rapid, but it does not give constant results. It should be confirmed by some other method unless a definite reaction to one of the fixed type sera has been obtained.

Neufeld's "Quellung" reaction.—Neufeld first observed that when the pneumococcus is treated with its homologous serum, the capsule of the organism becomes greatly swollen, the so-called "Quellung" reaction. This swelling affects also the capsular

substance between the two elements of the diplococcus, so that they are more widely separated. This swelling does not occur in mixtures with heterologous sera. This phenomenon has been utilized to determine the type of pneumococcus directly from the sputum, by mixing a loopful of the sputum with an equal quantity of undiluted rabbit serum of each of the types, and a loopful of Löffler's methylene blue. Hanging drop preparations are made and examined with the oil-immersion lens. The phenomenon may be observed within two or three minutes in the preparation mixed with the homologous serum. Immune *rabbit* serum must be used. The reaction is not given by the usual diagnostic or therapeutic sera which are obtained from horses.

Valentine (1933) allows the sputum and sera mixtures to stand for 20 to 30 minutes on the slides, covered with cover slips, in a moist chamber to prevent drying. The cover slips are then removed and the smears washed gently to remove the serum. After staining with dilute carbol fuchsin and then methylene blue the pneumococci treated with the homologous serum show a red capsule, whereas the others remain unstained.

Demonstration of specific soluble substance in urine.—Type determinations can be made by layering 0.5 cc. of perfectly clear urine over an equal amount of serum of each of the three fixed types. A positive reaction is indicated by a faint cloud or a heavy flocculent precipitate. If it is negative, concentration may be attempted. Acidify 25 cc. of urine with acetic acid, boil down to 5 cc. and filter. Add this to 10 volumes of 95% alcohol, collect the precipitate, which contains the specific soluble substance, by centrifugalization, dry, redissolve in 3 cc. of salt solution, and centrifugalize until clear. This concentrates the specific soluble substance, and frequently gives a precipitate with the immune sera when the unconcentrated urine fails to do so. The reaction is present in about 65% of cases of pneumonia due to the fixed types. The specific soluble substance may appear in the urine 12 hours after the initial chill. It is nearly always demonstrable in the urine in cases of sepsis.

Epidemiology.—Pneumococci are present in the mouth in about 40%e of normal individuals. Before type differences were recognized it was supposed that pneumonia was due to an autoinfection with these mouth organisms. Type determinations have shown, however, that less than 2% of these mouth organisms belong to Types I and II, the epidemic types, whereas over 80% of the pneumococci isolated from lobar pneumonia belong to these types. These figures suggest that these organisms may be spread by convalescents or contacts who have been shown to harbor virulent fixed-type pneumococci for several weeks. It is conceivable also, in view of the recent work of Griffith and others on the dissociation of pneumococci, that fixed types of pneumococci may develop in the body from the relatively avirulent mouth forms.

The fact that these carriers do not develop pneumonia is one indication of the substantial natural immunity possessed by man even for the fixed types. The mechanism of this natural immunity is not known, but is probably complex. That substances in the serum play some part is suggested by the finding of Clough that serum from many normal individuals will protect mice from one or other of the fixed types. Although this protective power was nearly as effective in some instances as that of the homologous immune serum, the normal serum contained no demonstrable agglutinins, bacteriotropins or precipitins comparable to those of immune sera. Whatever the mechanism of protection in these normal sera, it seems probable that it is one factor in the natural immunity to pneumonia.

Serum treatment.—Therapeutic sera have been prepared to Types I and II, and also to Cooper's Type VII by injecting horses intravenously, first with dead organisms and

then with living cultures of high virulence. With the older unconcentrated sera large doses were used and violent reactions were common. Somewhat better results were obtained with the pneumococcus antibody solution of Huntoon, but reactions to it were still severe. Felton has developed a much more satisfactory preparation by concentrating the active substances and eliminating most of the inert protein. The antibodies are precipitated out of the serum with that fraction of the globulin which is precipitated by dilution with water at a pH of 6.6 to 6.8. This precipitate can be redissolved in one tenth the original volume with only slight loss of antibody. The potency of the solution is determined by mouse protection tests and expressed as units, one of which is the quantity of antibody solution which will protect mice from 1,000,000 M.L.D. of a virulent culture. This solution is given intravenously in doses of 10,000 to 20,000 units, preferably about 50,000 (or even 100,000 units, Cecil) during the first 24 hours, and continued in smaller quantities until clinical improvement is evident.

The adequacy of the dosage can be checked by slide agglutination tests. Serum is obtained from the patient 2 or 3 hours after each injection and mixed with a suspension of the type of pneumococcus involved. Complete agglutination indicates that sufficient serum has been given.

Reactions to the injection, if properly given, are infrequent and relatively mild. The martality in Type I cases treated with Felton's antibody solution in the Bellevue, New York and Harlem Hospitals was 30% to 42% less than in the corresponding control series not receiving serum. The mortality in Type II was also reduced though to a lesser degree. The antibody solution now furnished is bivalent, active for both Type I and Type II pneumonias. Since the results are much better when the scrum is given early (within 72 hours) (11.5% mortality as compared with 20% in Cecil's series) it is advisable to give the first injection without waiting for a type determination. The latter should always be carried out and serum treatment continued only in Type I and Type II cases. No preparation has yet been produced which is effective in Type III cases.

Before giving serum or antibody solution one should give intradermal or preferably conjunctival tests for hypersensitiveness to horse serum. (See Hypersensitiveness, p. 253.) Recent investigations, particularly by Horsfall and Goodner (1936) have shown that antipneumococcus serum obtained from rabbits differs in many important respects from that obtained from horses. The antibody content in rabbit serum is higher, as measured by protective power for mice, and a much higher degree of concentration can be obtained. The size of the antibody molecules is much smaller in rabbit serum, the protective substances are more readily diffusible and they appear to reach the infecting organisms in the lesions more effectively. Rabbit antipneumococcus serum does not show the prozone phenomenon (inhibition of protective power by an excess of antibody) manifested by horse antiserum. Although rabbit antipneumococcus serum has not yet been given an extensive clinical trial, it appears to possess so many advantages over horse serum that it is likely to replace the latter as a therapeutic agent.

Prophylactic vaccines have been tried extensively, but the results have been inconclusive.

#### SARCINA FORMS

These are best observed in hanging-drop preparations, in which they can be seen as little cubes, like a parcel tied with a string. By noting them when turning over, it will

be seen that they are different from the tetrads which divide in only two dimensions of space. At times the packet formation is not perfect and it is difficult to distinguish such as sarcinae. All sarcinae are Gram-positive. If the staining of sarcinae be too deep it may obscure the lines of cleavage.

Various sarcinae have been isolated from the stomach, especially when there is stagnation of stomach contents. Sarcinae have also been found in the intestines. In plates the S. lulea is frequently a contaminating organism, since it is often present in the air. The demonstration of sarcina morphology should always be made from liquid media.

#### GRAM-POSITIVE COCCI

It is convenient to divide all cocci which do not show chain or packet formation into two classes, namely, those which are Gram-positive and those which are Gram-negative.

Gaffkya tetragena (M. tetragenus) Gaffky, 1881.—This organism is frequently found associated with other organisms in sputum, especially with tubercle and Pfeisfer bacilli. Smears from sputum or pus show a large coccus arranged in fours and surrounded by a broad capsule. In cultures the capsule is often absent. The colonies, which are rather slow-growing, are white, slightly smaller than those of staphylococci and are quite viscid. It does not liquefy gelatin but produces acid in glucose, lactose, saccharose and mannite. Milk is slightly acidified, and is usually coagulated in 1 to 3 days but the coagulum is not digested. It may be responsible for abscesses about the mouth, especially the teeth. Injected subcutaneously into Japanese mice, it produces a fatal septicaemia in three or four days and the blood shows great numbers of encapsulated tetrads. It has been reported as a cause of septicaemia in man.

### STAPHYLOCOCCUS INFECTIONS

Staphylococci (Ogston, 1881).—The staphylococcus is a round, Grampositive coccus which divides irregularly into masses which have been likened to clusters of grapes. They are grouped roughly into three types according to their pigment production; Staphylococcus albus, Staphylococcus aureus, and Staphylococcus citreus, although this characteristic is a variable one. S. albus is commonly found on the skin, and in the nose and throat. It is occasionally pathogenic. A variety called S. epidermidis has been found in stitch abscesses. S. citreus produces a lemon-yellow pigment in culture, and is only feebly pathogenic. S. aureus produces a golden yellow colony, and is the common cause of various suppurating conditions.

Cultural characteristics.—Staphylococci grow readily at room temperature but better at 37°C., on all the ordinary media. They acidify and coagulate milk, liquefy gelatin, and produce a uniform turbidity in broth. Glucose, lactose, and saccharose are fermented with the production of acid, but not gas. Coagulated serum media are slightly liquefied. Pigment production is especially abundant on potato. On blood agar there is a variable amount of haemolysis around the colonies. Staphylococci are exceptionally resistant to desiccation, and cultures or dried pus may contain viable organisms for

months. They are more resistant to heat than most vegetative forms of bacteria, and temperatures of 58° to 60°C., for one half hour may be required in the preparation of heat-killed vaccines.

Variation.—Hoffstadt and Youmans have dissociated smooth S. aureus cultures into rough forms and have obtained the small G. type of colony composed of minute filtrable forms. These dissociated forms differed from the original in a lack of pigment production, and of pathogenicity.

Toxins.—S. aureus and even some strains of S. albus produce, in varying amounts, certain toxins. Filtrates of old broth cultures contain a ferment-like substance, leukocidin, which disintegrates leukocytes. Many of the virulent strains also elaborate an haemolysin which may be demonstrated in vivo or in vitro. A natural anti-leukocidin and antihaemolysin may occur in normal blood and can be produced in animals by injection of the toxin. A tissue-necrotizing toxin has been found which produces a local lesion in the skin, akin to an abscess, on intradermal injection. Certain strains elaborate a lethal toxin which causes sudden death on subcutaneous or intravenous inoculation. Whether these activities are different manifestations of the same toxin or of distinct substances is unsettled. Outbreaks of food poisoning due to a staphylococcus toxin from contaminated food have occurred.

Pathogenicity.—S. aureus, and occasionally virulent strains of S. albus are the common cause of boils, abscesses and carbuncles, and other skin infections. Infections of the tonsils, sinuses, middle ear, and mastoid are frequently caused by staphylococci. From such infections the organisms may get into the blood stream and cause a septicaemia or pyaemia with multiple secondary abscesses in the kidney, liver and other organs. Endocarditis and suppurative lesions in the joints may occur. This clinical picture can be produced in rabbits by intravenous injection. Osteomyelitis is most frequently caused by the staphylococcus, which is carried to the bone by the blood stream from a distant focus. Pyelitis and pyelonephritis may be due to these organisms. Long-continued infection may result in amyloidosis. Impetigo contagiosa and pemphigoid eruptions in children are often staphylococcal. In the tropics, where resistance is often lowered and skin infections are common, these infections often show great virulence, and long continued fevers are often staphylococcal septicaemias. Invasion of the blood stream by staphylococci occurs frequently as a terminal event or after death, so that they are often found in post-mortem blood cultures.

S. albus is a common contaminant of blood cultures, and when found must be regarded with great scepticism. However, it does (rarely) cause fatal septicaemia.

Immunity. - Injection of staphylococci into animals cause the formation of demon strable antibodies including (with some strains) antitoxins. Attempts have been made to separate staphylococci into groups by means of agglutination tests. Such groups, however, are not well defined, and do not correspond in virulence or pigment production.

Julianelle and Wieghard (1934, 1935) isolated from staphylococci a complex antigenic protein common to all types, and two specific polysaccharides (haptenes), one common to the pathogenic strains, and the other present in the saprophytic strains. These can be identified by means of precipitin reactions with the serum of rabbits immunized by injections of the whole organisms. In general, the pathogenic strains produce pigment, form haemolysins and other toxins, and actively liquefy gelatin and ferment carbohydrates. The non-pathogenic strains lack these properties or possess them in lesser degree. There are many intermediate strains, and the two groups can not be sharply differentiated from each other.

Treatment.—Vaccines, toxin, or toxoid preparations are useful in the treatment of some staphylococcal infections. Autogenous vaccines seem to be more effective than those prepared from old stock cultures. This may depend upon strain differences and also upon the fact that freshly isolated strains are not dissociated, and are, therefore, more antigenic. Intracutaneous reactions to injections of toxin are used as a test of susceptibility and an indication for treatment with toxin. Serum therapy hitherto has been useless. Recently, however, antitoxic sera have been obtained which exert a definite protective action in animals and which appear to be of some value in human infections.

Bacteriophage therapy has seemed to be of value in the treatment of some staphylococcus skin infections and of cystitis. It is injected into the affected area and also applied locally in the form of wet dressings.

## GRAM-NEGATIVE COCCI—NEISSERIA GROUP

The organisms in this group are Gram-negative diplococci which are characteristically flattened at their opposed surfaces so that they are shaped like a biscuit or coffee bean. Two species are of great importance, the gonococcus and the meningococcus. Other members of the group, N. catarrhalis, N. flava, and N. sicca, are important chiefly because they may be confused with the two pathogenic species.

## GONOCOCCAL INFECTIONS

Neisseria gonorrhoeae (gonococcus) (Neisser, 1879).—The gonococcus is the cause of gonorrhoea, gonorrhoeal ophthalmia, and occasionally of systemic manifestations such as gonorrhoeal arthritis, endocarditis, and septicaemia.

Morphology.—The organism is typically plano-convex or biscuit-shaped, although in old cultures or in secretions from chronic cases there is a tendency to involution forms. In the secretions they are generally found grouped in masses of several pairs, most characteristically within the pus cells, or on epithelial cells, but they are also found extracellularly. They can usually be identified readily with a Gram stain.

Cultural characteristics.—The gonococcus grows only at temperatures of from 30° to 39°C. in enriched media which are sufficiently moist. It will not grow on plain media unless considerable pus is carried over in the inoculation. (For special media see p. 847.) On serum or hydrocoele agar, or blood agar, the colonics appear as small, discrete, dewdrop spots, at first round, and later showing a slightly irregular margin. Cultures die out within 4 to 10 days unless subcultured on suitable media. The gonococcus produces acid in glucose but not in maltose, while the meningococcus produces acid in both.

Viability.—The organism is killed in 5 hours by a temperature of 45°C, and speedily by drying. In moist smears of pus it may live for 1 or 2 days.

Serological differentiation.—Differentiation of gonococci has been attempted by different investigators by means of agglutination tests, and a number of ill-defined groups have been found.

Pathogenicity.—Animals do not contract true gonorrhoea, although local necrosis with some systemic reaction occurs on subcutaneous or intravenous inoculation, and immune sera can be produced.

Gonorrhoea is the commonest of the venereal diseases. During the World War almost 6% of the recruits had venereal disease when taken into the army, and most of the cases were of gonorrhoea. Its great importance from the public health standpoint lies in the fact that an individual may remain infectious for years after the symptoms subside. In the male the gonococcus infects the urethra, and may invade the periurethral tissue, the prostate, seminal vesicles and epididymis.

Laboratory diagnosis is made by demonstrating the gonococci in smears from the urethral discharge. In the acute stage, when the discharge is

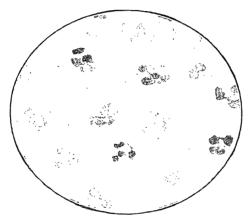


Fig. 3.—Gonococcus. Film from urethra.

abundant, they are found in large numbers, chiefly in the pus cells; later on, in the chronic cases, the pus cells largely disappear, and the organisms are found in smaller numbers on the epithelial cells. Secretion must be obtained by massage of the prostate and seminal vesicles.

Exercise, eating stimulating food, and if necessary the passage of a sound are useful as a preliminary. The glans and meatus should be cleansed, and part of the urine is voided to wash out the urethra. The prostate is then massaged in the usual manner, and the secretion collected in a sterile dish. The rest of the urine is then voided into a sterile flask. Cultures and stained films are made from the prostatic secretion and the sediment from both urine specimens. These will usually contain also any organisms present in the urethra. In such smears gonococci are often atypical in their morphology with many distorted shapes and involution forms. In urinary sediments Gram-negative coccoid forms of colon bacilli may be present which may be intracellular, and thus may be mistaken for gonococci. It is, therefore, important to continue the search until some

fairly typical diplococci are found. There is nothing requiring greater discrimination than a diagnosis from such a smear.

Cultures.—By using a special chocolate agar, by incubating in an atmosphere of 8% CO<sub>2</sub> and by using the oxidase reaction as a means of recognizing the colonics (see p. 848), McLeod et al. were able to isolate the organism in many cases in which they were not found in stained smears. In a series of 275 cases, cultures were positive in 42%; smears were "almost certainly positive" in 20%, probably positive in 20%. The certainty of the diagnosis when made by culture makes this method much superior to smears, particularly in clinically questionable cases.

In the female the favorite sites for the organisms are the urethra and the cervix uteri. From here the infection may spread to the tubes, ovaries and pelvic peritoneum. It is one of the commonest causes of sterility in males as well as females. For diagnosis separate smears should be made from the urethral meatus and from the cervical canal. The vagina of the adult does not provide a suitable soil for the development of gonococci. In female children, however, the gonococcus produces a vulvo-vaginitis, which is extremely contagious, and in hospitals and institutions may spread with great rapidity. These epidemics are very difficult to control, and the treatment of infected children may require many months before they are bacteria-free.

It has been claimed by some investigators (Pearce) that the organisms found in children were serologically distinct from those from adult cases, but this claim has not been substantiated by others. The different manifestations in children appear to be due to the immature mucous membrane of the vagina, upon which the organisms flourish. Lewis and others have had good results in treating these cases by the injection of the ovarian hormone, theelin, which causes a change in the infantile mucous membrane of the vagina to the adult type.

Gonorrhoeal ophthalmia.—One of the greatest advances in preventive medicine was the Credé method of instilling silver nitrate into the eyes of the new born, thus preventing ophthalmia and blindness in those born of gonococcus-infected mothers. In the adult the eye may occasionally become secondarily infected. The diagnosis is readily made by smears from the exudate.

Acute gonococcal arthritis may occur in an infected individual. It is often monoarticular, or, if polyarticular, one joint may be more severely affected than the others. In fluid from such joints, which may be sero-fibrinous or purulent, gonococci may be demonstrated by smears and in culture. In the sero-fibrinous fluids the organisms are more likely to be found in the flecks of fibrin. Although infection of the joints is metastatic through the blood stream, positive blood cultures are rarely obtained

except in a small number of cases which develop an acute endocarditis similar to that caused by the streptococcus.

Complement fixation test.—This test is of value, particularly in the differentiation of gonorrhoeal arthritis from that due to other causes. In preparing the antigen a number of strains of gonococci should be used since serological differences have been demonstrated.

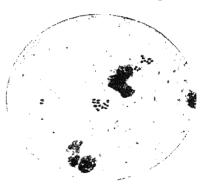
Treatment.—Local treatment should be carried out until at least three consecutive smears are negative for gonococci. Vaccines (preferably autogenous) may be of some value in gonorrhoeal arthritis, but have little if any effect in the ordinary case of specific urethritis. Sulfanilamide exerts a marked curative action on gonorrhoeal infections. The urethral discharge is rapidly diminished, and infection of the posterior urethra and prostate can usually be prevented by its administration.

## EPIDEMIC CEREBROSPINAL MENINGITIS AND MENINGOCOCCAEMIA

Neisseria intracellularis (Diplococcus intracellularis meningitidis) Weichselbaum, 1887.—This is the cause of about 70% of the sporadic

cases of acute cerebrospinal meningitis, and of almost all of the epidemic cases. The organisms are present in the cerebrospinal fluid and are often demonstrable in the blood early in the disease.

The meningococcus may cause sepsis characterized by high fever, purpura, and often haemorrhages into the adrenals with profound shock, without signs of meningitis (Waterhouse-Friderichsen syndrome). Death may occur within 24 hours. Early recognition and prompt treat- Fig. 4.—Neisseria intracellularis and pus ment are essential.



cells. (X1000.) (Williams.)

Morphology.—The meningococcus is a small, Gram-negative, biscuit-shaped diplococcus, and, like the gonococcus, it is found chiefly within the leukocytes in the cerebrospinal fluid. There is a greater tendency to variation in size and shape than is the case with the gonococci, which, in fresh material, are more uniform.

Cultural characteristics.—The meningococcus usually requires enriched media for growth from body fluids. On blood agar, smooth, moist, slightly hazy colonics appear after twenty-four to forty-eight hours. They are usually about 1 or 2 mm. in diameter although they may be much larger. Later the center may become somewhat opaque and raised. Unless considerable spinal fluid is transferred to the culture, meningococci will not grow on plain agar. They grow best at 37°C. although growth may be obtained between 25°C. and 42°C. They ferment glucose and maltose, with the production of acid, but not lactose or saccharose.

Viability.—The meningococcus is very sensitive to light, cold, and desiccation. Material for culture, therefore, must be kept warm, and cultured on warm, moist slants as soon as possible in generous quantities. Cultures when first isolated die out very rapidly—within two or three days, so that it is necessary to make frequent transfers to keep the organisms alive. Cultures are best kept at  $37^{\circ}$ C. on blood agar or egg yolk slants. Branham, however, has been able to keep her stock cultures viable for at least two years by keeping them frozen at  $-15^{\circ}$ C.

Variation.—Rake (1933) has described variants of the meningococcus arising secondarily, after thirty-six hours, from the center or the periphery of the characteristic smooth colonies. These secondary growths were thicker, drier, and more opaque than the original, and, when subcultured, they resembled old stock laboratory strains in morphology, cultural characteristics, and viability. He has found both rough and smooth colonies in cultures from the spinal fluid and has produced rough strains in cultures from smooth strains. The rough strains showed a tendency to spontaneous agglutination or to non-specific agglutination in the immune sera. Neither the smooth nor the rough forms were entirely stable; both tended to pass over into what he terms the "stock variant."

Pathogenicity.—The meningococcus is only feebly pathogenic for laboratory animals, with the exception of the mouse and the guinea pig. If the culture is suspended in a solution of mucin and injected intraperitoneally, the virulence for mice is greatly increased, so that as few as ten organisms may be fatal (Miller et al., 1936). Mice so infected may be protected by specific antiserum, and afford a satisfactory means of standardizing the scrum.

Scrological differentiation.—Dopter first called attention to the existence of two types which differed immunologically—the normal and the parameningococcus. Gordon grouped all the strains which he isolated into four different types. Of these cases he found that 40% fell into Type I; 45% into Type II; 10% into Type III; and 5%, into Type IV. The types found in patients and in carriers showed a close correspondence. He noted a close affinity between Types I and III, and between II and IV. Flexner and his associates in this country recognize two main types, the normal (Gordon Type II) and the parameningococcus (Gordon Type I) and in addition a number of heterogeneous strains. Branham has also shown that there is a considerable overlapping of these types in their serological reactions. According to this investigator (1037), Types I and III (A) have become practically indistinguishable and really constitute a single group (I–III). Types II and IV (B), on the other hand, have remained distinct from I–III and from each other. In recent epidemics the relative incidence of I–III has increased, constituting 85% of the cases in 1028–30 and 05% in 1034–30.

Miller and Boor (10,34) have described antigenic relationships between the nucleo proteins and polysaccharides of the meningococcus, gonococcus, and the type III and rough pneumococci.

Agglutinating sera for type determinations can be prepared by the intravenous inoculation of rabbits with increasing doses of dead or living cultures of the different types.

Diagnosis.—Blood cultures should always be made as soon as possible and repeated if there are signs of sepsis. As soon as the first signs of meningitis appear, a lumbar puncture should be done, and cultures from

the fluid should be made immediately upon suitable media in generous quantities. The remainder of the fluid is centrifugalized and the sediment is smeared and stained with methylene blue and by Gram's method.

A convenient method of culturing is that of Conradi. After centrifuging the spinal fluid, the clear upper fluid is poured into a test-tube, warmed to  $45^{\circ}$ C., then three times this amount of melted nutrient agar ( $45^{\circ}$ C.) is added, and the mixture poured into a plate. The surface of the plate is then smeared with the sediment from the centrifuge tube. Growth at first is scanty, later more vigorous. Plates must be kept at  $37^{\circ}$ C., and moisture maintained. Daily transfers are necessary at first. When the fluid is very cloudy the organisms can often be demonstrated in the fluid without centrifugalization. The meningococci are found chiefly within the leukocytes, but they may be extracellular. Occasionally, when the spinal fluid is almost clear, it may be impossible to demonstrate them either by smears or by cultures. At times they may be found either in smears or in cultures but not by both, so that both procedures are necessary for a thorough examination. In purulent fluids the meningococci may be autolyzed if the examination is not made promptly, whereas other organisms are more resistant to autolysis.

Pneumococci or streptococci in the spinal fluid are easily differentiated from meningococci by the Gram stain, as well as by culture. The influenza bacillus can be distinguished by its morphology; filamentous forms are common in the spinal fluid. Tuberculous meningitis may show a cloudy fluid, but the cells are chiefly lymphocytes. Acid-fast stains should be made on such fluids. Exceptionally, fluids from cases of encephalitis and poliomyclitis may be cloudy, and no organisms are demonstrable by smears or by culture. However, a frankly purulent fluid which contains no organisms is usually due to a meningococcus meningitis and should be treated immediately as such. In the naval training stations an occasional case of meningococcus infection, without meningismus, appears in a group of catarrhal fever (influenza?) patients. Instead of the normal or sub-normal white count of catarrhal fever we have a leukocytosis, often marked, and this finding is very important in the early diagnosis of meningitis.

Precipitin Test for Meningococi.—Vincent has recommended a precipitin test for epidemic cerebrospinal meningitis which has the advantages of being simple, of giving information sooner than cultures and of possessing particular value in those cases in which meningococci cannot be found in the smears or in cultures from the cerebrospinal fluid. It is performed by adding 1 or 2 drops of antimeningococcic serum to a tube of fresh cerebrospinal fluid which has been cleared by centrifugalization for ten to fifteen minutes. After adding the serum the tube is placed in the incubator at 52°C. for two to five hours together with a control tube. The formation of a precipitate (turbidity) shows a positive test. The test is more delicate if the fluid is layered over the serum. Alexander (1937) found that the appearance of a definite precipitate within ten minutes had an unfavorable significance. In a series of serum-treated cases the mortality was 77% in 22 positive cases but only 4% in 47 negative cases.

For clinical purposes the demonstration of a Gram-negative intracellular diplococcus in the spinal fluid is usually sufficient evidence that the case is one of meningococcus meningitis. For further identification of

the organism, however, it is necessary to determine its growth on culture media, including its fermentative abilities, and also its agglutinability in a polyvalent immune serum prepared from different strains including the main types.

Macroscopic agglutination.—Prepare a series of dilutions of an immune polyvalent serum from 1-25 to 1-400 or higher according to the titer of the serum. Place ½ cc. of each dilution into a series of small tubes and add to each an equal volume of a homogeneous suspension of the organisms. This is prepared by washing off an 18 to 24 hour blood or serum agar culture with enough salt solution to give a definite but rather faint turbidity. A control tube containing normal horse serum (or salt solution) instead of the immune serum is essential. Incubate for 16 to 24 hours in a water bath at 56°C. The organism is identified as a meningococcus if clumping occurs in the higher dilutions of the immune serum and not in the control tube.

To determine the type of meningococcus, another series of tubes is set up containing similar dilutions of monovalent serum of each type. If the results are not clear cut, as is frequently the case, agglutinin absorption tests can be carried out. Even by this method, however, Branham finds considerable over-lapping of the strains.

Throat cultures.—Cultures from the nasopharynx are used chiefly for the detection of carriers. A bent wire applicator with a sterile cotton tip is introduced behind the soft palate and swabbed over the naso-pharynx. The material obtained is immediately spread over a blood agar plate which should be warmed previously to body temperature. Suspicious colonies are subcultured the following day, and examined microscopically. Other Gram-negative diplococci, N. calarrhalis, N. flava and N. sicca, may be present in the normal pharynx and must be differentiated by their cultural characteristics and by their agglutinability in normal horse serum. From these subcultures macroscopic agglutination tests with the polyvalent serum are made as described above.

For a rapid examination of colonies direct from the plate Houghton has modified the slide agglutination method of Krumwiede as follows: Take a large loopful of 1 to 10 normal horse serum. Emulsify the colony from a plate in this serum. If agglutination occurs it is not a meningococcus. If agglutination does not occur he rubs in a small loopful of polyvalent serum (undiluted). The curdy clumping which begins to show itself within a minute or so is distinctly observed with the naked eye, or better with a magnifying lens, or the  $\frac{2}{3}$ -inch objective. After the preparation dries make a Gram stain. By successively using small loopfuls of type sera, before adding the polyvalent, one can quickly obtain evidence as to type to be later verified by macro scopic agglutination.

Epidemiology.—The meningococcus is transmitted by direct contact either with cases of meningitis, or, more frequently, with healthy carriers. Persons in contact with cases of meningitis or with healthy carriers may show a high incidence of carriers, 10% or even higher. In military camps from 2 to 5% of the incoming recruits have shown meningococci in nasopharyngeal cultures. Most cases cease to be carriers within a few weeks, but in some cases the carrier period may persist for months. It is these chronic carriers which are responsible for the sporadic cases. It is impossible at present to determine the virulence of strains from the nasopharynx. Frequently they show atypical agglutination reactions. Rake (1933) has found that a majority of the strains which he obtained from the nasopharynx resembled the "stock" variants, and not the

#### SERUM TREATMENT OF MENINGITIS

typical smooth type obtained from spinal fluids. Natural resistance to the disease is high, especially in adults, but there is no method of measuring susceptibility.

In military barracks suitable spacing between cots decreases the carrier percentage. Isolation and treatment of carriers is rarely practicable. During the World War the naval service discontinued meningococcus carrier examinations, as a routine, and this practice was shortly afterwards followed by the military authorities, without demonstrable increase in the incidence of the disease.

The main points in epidemiology as at present recognized are: (1) worldwide distribution; (2) uneven distribution of prevalence—epidemic or sporadic; (3) low attack rate and spread; and (4) high incidence in children and military and other aggregations.

It is an open question whether the meningococcus reaches the subarachnoid space from the nasopharynx through the lymphatics, or by way of the blood stream. It seems probable that it is primarily a blood infection, the meningococci localizing later in the central nervous system. Occasionally, the infection is confined to the blood stream, and the diagnosis must be made by blood cultures.

Serum treatment.—Therapeutic immune serum is prepared by the intravenous injection of horses with increasing doses of living meningococci. It is necessary that the serum for treatment should contain antibodies for the different types, and since many strains do not fall into these principal types, it is important to use, in addition, a mixture of a number of miscellaneous strains of different antigenic properties. Wadsworth uses six strains, which, however, are very carefully chosen. The National Institute of Health supplies representative strains to various biological laboratories, from which as many as twelve are used in producing the serum. Immunization requires at least three months. The serum from the immunized horses should agglutinate the normal and para types in a dilution of at least 1–1500. Polyvalent serum is used exclusively for treatment.

To administer the serum, withdraw spinal fluid until the pressure is relieved and the fluid comes out by drops at the rate of four or five per minute. Leaving the needle in place, inject serum (by the gravity method), which has been previously warmed to body temperature, equal in amount to one-half or three-fourths of the spinal fluid removed. This should be repeated according to the symptoms, and the character of the cerebrospinal fluid. Daily injections for from three days to a week are necessary in the average case. In addition, one should give from 50 to 100 cc. of serum intravenously, and continue with intravenous therapy at least as long as organisms are present in the blood stream.

The results of serum therapy are best when it is given early in the disease. When given before the third day the average mortality reported by several observers was under 12%. It is advisable, therefore, to administer serum at the first lumbar puncture, if the fluid is turbid, without waiting for identification of the organism.

Antitoxin.—Ferry et al. (1933) obtained bacteria-free filtrates of cultures of the four types of meningococci which contained soluble toxins (specific for each type), and with these they produced potent antitoxic sera. By intraperitoneal injections of antitoxin he was able to cure monkeys with experimentally induced meningitis. Hoyne (1935) reported favorable results in the treatment of meningitis by combined intravenous and intraspinous injections of antitoxin. In a series of 85 cases treated with antitoxin, the mortality was 23.5%, whereas in a control series of 211 cases treated with antimeningococcic serum the mortality was 45.9%. More recently (1936) Hoyne reported favorable results in a small series of cases treated only by intravenous injections of antitoxin, without lumbar puncture.

Susceptibility to drugs.—Meningococci are very sensitive to the action of prontosil (sulfanilamide). This drug may protect mice from 1,000,000 M. L. D. of culture suspended in mucin and injected intraperitoneally. Such favorable results have been obtained clinically that combined treatment with serum and sulfanilamide seems advisable. Schwentker et al. (1937) have recently reported a small series of cases treated with sulfanilamide alone, with a mortality of 9%.

Carey and Pincoffs (1937) have observed one case of fulminant sepsis (Waterhouse-Friderichsen syndrome) which recovered after energetic treatment by means of injections of adrenal cortical extract, sulfanilamide and antimeningococcus serum.

Neisseriu Flavescens.—Branham, in 1930, reported an epidemic of cerebrospinal meningitis in Chicago caused by this organism. There were 14 cases of which 4 died. The organism produced a golden yellow pigment in cultures, and the colonies were less moist than those of the ordinary meningococcus. None of the differentiating sugars (dextrose, levulose, maltose and saccharose) were fermented.

Neisseria catarrhalis (Micrococcus catarrhalis) Seifert, 1890.—This organism has been specially studied by Lord. It resembles the Meningococcus strikingly and can be differentiated only by agglutination and cultural procedures. It grows on plain agar and at room temperature. It not only occurs in the nasal secretions of healthy people, but appears to be responsible for certain coryzas and bronchial affections resembling influenza. It is responsible also for certain epidemics of conjunctivitis. It has been reported in a few cases as a cause of sepsis and endocarditis.

Original cultures may show only slight growth, whereas subcultures prove luxuriant. The colonies are larger, more opaque, and have a more irregular wavy border than the round colonies of the *Meningococcus*. The colony is usually easily picked up from the plate with the loop and does not emulsify readily. *M. catarrhalis* grows best at 37°C. but also fairly well at 22°C. after several days, while the *Meningococcus* requires body temperature. It does not ferment any of the sugars.

Neisseria flava (Micrococcus flavus).—This organism is somewhat smaller than the meningococcus. The colonies have a yellowish center and adhere somewhat to the medium. There are three types of this organism, differentiated by fermentation reactions. It is well to remember that N. flava will agglutinate with 1-50 normal horse serum so that in meningococcus agglutinations one should run a normal horse serum control, the meningococcus agglutinating only with immune serum.

	Glucose	Maltose	Saccharose	Levulose
Meningococcus	+	+		_
Parameningococcus	+			
N. flavescens			_	
N. gonorrhoeae	+	-		
N. flava I	+	+	+	
N. flava II	+	+		+
N. flava III	+	+		
N. catarrhalis	_	_ '		
N. sicca	+	+	+	+

**Neisseria sicca** (*Micrococcus pharyngis siccus*).—This is a small coccus which forms white colonies, which are firm, stick to the medium and do not emulsify easily. Growth takes place at room temperature.

Gordon has considered the ability of the meningococcus-like organisms to grow at lower temperatures than the "normal" and "para" strains of meningococcus as being of differentiating value.

The table on page 52 gives the fermentation reactions of the various Gram-negative cocci.

## CHAPTER III

# STUDY AND IDENTIFICATION OF BACTERIA—SPORE-BEARING BACILLI. KEY AND NOTES

- A. Grow Aerobically.—Mostly saprophytes. Generally liquefy gelatin and digest coagulated blood serum. Often occur in long chains and form rhizoid colonies. Usually Gram-positive. Form of rod usually not greatly changed at sporulation. (Type species *Bacillus subtilis* Cohn.)
  - (a) Pathogenic forms.
    - (r) Bacillus anthracis. Non-motile rods with square cut to concave ends, occurring in long chains. Central spores.
  - (b) Non-pathogenic forms. Usually motile, having central or excentric spore. Facultative aerobes.
    - (r) Bacillus subtilis group. Organisms of this group are commonly found in intestinal contents, soil, water and milk. Seventy-five different species described, showing slight cultural differences. Many produce pigment.

Non-pathogenic Spore-bearing Aerobes on Agar
Table of Gruner and Fraser (modified).

Surface dry

Gray-white
Edges feathery
B. mycoides.

Surface gummy
White
B. mesentericus.

Yellow
B. ruminatus.

Dirty gray
B. graveolens.

White and
Wrinkled.

Non-pathogenic Spore-bearing Aerobes on Agar

Resource Horbston Agar

Agar

B. subtilis (motile).

B. ellenbachiensis (non-motile).

B. mycoides.

Yellow
B. ruminatus.

Dirty gray
B. graveolens.

White and
Wrinkled.

NOTES.—B. subtilis has square ends and central spores, which do not cause bulging B. vulgatus is long and slender. Slightly oval spores.

- B. mesentericus varies. Usually short with rounded ends and central, bulging spores.
- B. ellenbachiensis has rounded ends, oval spores and shows granule formation (beaded)—resembling diphtheroids.
  - All of these grow well at room temperature, optimum 30°C.
- B. Grow Only Anaerobically.—Producing clostridium (spores) forms. Often parasitic. Many elaborate exotoxins.
  - (a) Motile. Rods swollen at sporulation.
    - (a) Spores oval, central or excentric.
      - (1) Clostridium chauvei. (Called the bacillus of symptomatic anthrax; cause of black leg or "quarter evil" in cattle.)
      - (2) Clostridium oedematis-maligni (Vibrion septique, the cause of malignant oedema).

55

- (3) Clostridium oedematiens. (Gas-oedema bacillus of Aschoff.)
- (4) Clostridium botulinum, Type A. (The cause of botulism. Filtrate toxic for guinea pigs and chickens.)
- (5) Clostridium botulinum, Type B. (Differs from Type A in toxicity. Non-toxic for chickens. Exotoxin not neutralized by Type A anti-toxin.)
- (6) Clostridium histolyticum. (Isolated from war wounds. Produces necrosis in tissues.)
- (7) Clostridium sporogenes. (Isolated from intestinal contents. Indicates faecal pollution of water.)
- (8) Clostridium fallax. (B. fallax. Feebly pathogenic.)
- (b) Spores terminal or subterminal. Spherical or nearly so.
  - (9) Clostridium tetani. (Cause of tetanus. Forms highly poisonous exotoxin.)
- (c) Spores oval or elongated.
  - (10) Clostridium tertium. (Isolated from war wounds.)
- $(\beta)$  Non-motile. Rods not swollen at sporulation.
  - (a) Spores central or excentric. Encapsulated.
    - Clostridium welchii. Types I, II, III, IV. (Cause of gas gangrene. Types differentiated by fermentation reactions.)
    - (2) Clostridium egens. (Similar to Cl. welchii.)

#### SPORE-BEARING AEROBES

#### Anthrax

Bacillus anthracis (Discovered by Pollander, 1849; its nature recognized by Davaine, 1863, and proved by Koch, 1876).—This is the only aerobic spore-bearing bacillus of medical importance.

Morphology and cultural characteristics.—This organism is a Gram-positive bacillus 5 to  $8\mu$  by 1 to 1.5 $\mu$ . In cultures it has square-cut or concave ends and is often found in long chains, whereas in the blood of an infected animal it is in quite short chains, and the ends may be slightly swollen. It is non-motile. A capsule may be demonstrated in preparations from the animal body. The spores are oval and centrally placed, and stain with difficulty.

B. anthracis grows well on all media and rapidly liquefies gelatin. On a gelatin plate at about the time liquefaction begins, the colony has a characteristic appearance resembling a medusa-head. Milk is slowly acidified and coagulated. Spores develop most readily on potato media at a temperature of 30°C., and do not form at temperatures above 43°C. They are not formed in the blood or tissues (in the absence of oxygen).

Methylene-blue reaction of McFadyean.—This reaction is useful in the recognition of anthrax bacilli in blood films. The films are made in the usual way, dried, and flamed three times. Stain with polychrome methylene blue, wash, and dry. A characteristic amorphous, purplish material is seen around the bacteria, which represents the disintegrated capsules.

Variation.—Variations in capsule formation and virulence occur. When the capsule is absent or small, the colonies are smaller and more mucoid than those of the encapsulated, rough forms. The smooth forms are less virulent. The attenuation of the organisms for vaccine by growth at high temperature causes a change from rough to smooth form.

Anthrax in animals.—Anthrax is an important disease in domesticated animals, especially sheep and cattle. The characteristic pathological lesion produced in animals is the greatly enlarged, friable, mushy spleen. The mortality is from 75 to 100%. Like cattle, guinea pigs, brown rats, mice, and rabbits are quite susceptible. Man is much less susceptible,

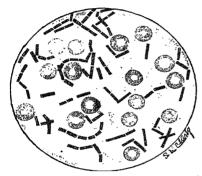


Fig. 5. -Bacillus anthracis in blood of rabbit. (Coplin.)

but more so than the goat, horse, and pig. The white rat and the Algerian sheep have a high degree of immunity.

Stiles thinks that animals are infected by eating the bones of animals dead of anthrax, which make a portal of entry for the organisms by piercing the buccal mucous membrane. Others think that the spores are swallowed by the animal and vegetative forms develop in the alimentary tract and invade the body through the intestinal wall. Although spores do not form in an intact body, they form in the presence of oxygen after a postmortem

or disintegration of the body by maggots. For this reason the diagnosis should be made without opening up the body, unless it can be burned immediately, by staining the organisms in films of blood from the ear vessels. Dried spores live for years and can withstand boiling for hours.

Immunization of animals.—The mechanism of the immunity produced in animals is not known. The injection of dead organisms seems to have no effect. In Pasteur's method, which has given excellent results, two vaccines are cultivated at 42.5°C. The first is attenuated for fifteen days, and the second for only ten days, and is given twelve days after the first. This immunity lasts for about a year. At this temperature only vegetative forms develop. Prolonged incubation destroys virulence for rabbits and then for guinea pigs and eventually for white mice.

Anthrax in man.—Men are chiefly infected by working with hides, wool, or meat of diseased animals. Many infections have been traced to shaving brushes. The organism has been cultured from them, especially from new brushes. Brushes may be sterilized by soaking for four hours

in 10% formalin solution at 110°C. Autoclaving at 15 pounds for three hours may be used for hair and bristles. Live steam at 100°C. kills the organisms in five to ten minutes. Horsehair from China and Thibet seems the chief source of infection. The two most important types, those due to handling products of diseased animals, are: (1) Malignant pustule, and (2) Woolsorters' disease. An intestinal type, with a high mortality, from ingestion of infected meat eaten raw, is also known.

Malignant pustule is due to inoculation of a cut or abrasion and, therefore, frequently occurs on the arms and backs of men unloading hides. It first appears as a pimple, and the center begins to show a vesicle, which changes into a black, necrotic area with a red, oedematous areola. If the



Fig. 6.—Anthrax bacilli. Cover glass has been pressed on a colony and then fixed and stained. (Kolle and Wassermann.)

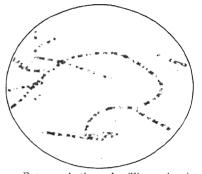


Fig. 7.—Anthrax bacilli growing in a chain and exhibiting spores. (Kolle and Wassermann.)

pustule is excised early, the prognosis is improved. In fatal cases in which the pustule is not excised, a postmortem does not show the enlargement of the spleen and the abundance of bacteria in the kidneys seen in animals. Death seems to be due to toxaemia rather than septicaemia. A few cases with positive blood cultures have been reported. A case has been reported by Graham which recovered after intravenous injections of anti-anthrax serum.

Woolsorters' disease.—This type is a severe pneumonia with a high mortality, characterized by an oedema of the lungs which is haemorrhagic about the bronchi, and by great swelling and oedema of the bronchial and mediastinal glands. It is believed to be due to inhalation of spores.

Laboratory diagnosis.—In taking material from a malignant pustule before excision, be careful to avoid rough manipulations, or bacteria may

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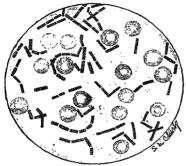


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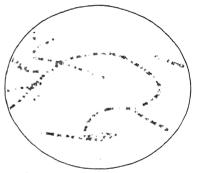


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Laboratory diagnosis.—In taking material from a malignant pustule before excision, be careful to avoid rough manipulations, or bacteria may

be expressed into the circulation. Make cover-glass preparations, using Gram's stain. Make a culture on agar. Blood cultures are rarely positive until late in the disease. Inoculate a guinea pig or mouse subcutaneously, the mouse at the root of the tail. A few bacilli are often sufficient to kill the animal.

The guinea pig dies in about forty-eight hours. It shows an oedematous, gelatinous exudate at the site of inoculation. The blood is black and swarms with anthrax bacilli. It is the best example of a bacteriaemia.

Detection in animal tissues. Precipitin test.—For the diagnosis of anthrax in decomposed tissues, Ascoli has devised a precipitin test. A piece of tissue is boiled in a few cc. of salt solution, which is then filtered until clear, and layered on the immune serum. In a positive test a precipitate occurs at the junction of the fluids. Suitable controls must be made, and a serum with known precipitating power must be used.

Demonstration of capsules in body fluids from decomposed animals is valuable in differentiating anthrax from other contaminating organisms.

For the detection of anthrax bacilli in shaving brush bristles or in wool, the materialis shaken vigorously with sterile salt solution. The solution is then decanted and centrifugalized. The sediment is resuspended in Icc. of salt solution and heated to 80°C. for a half hour to kill all non-spore-bearing organisms. This may then be cultured, and injected subcutaneously into an animal.

Serum Irealment.—In cases in which the infection becomes septicaemic instead of localized, the outcome is usually fatal. In such cases one should inject anti-anthrax serum intravenously, in doses of 50 to 75 cc. In malignant pustule it is advisable to inject the serum in the subcutaneous tissues surrounding the lesion. The anthrax antiserum is made by simultaneous inoculation of animals with cultures of B. anthracis and antiserum. The best animal to use is the sheep and a preliminary vaccination by Pasteur's method is to be carried out.

Various bacteria, especially the pyocyaneus bacillus, show marked antagonism to *B. anthracis*. Pyocyanase digests the anthrax bacillus and has been used to cure animals infected with anthrax.

B. anthracoides is an aerobic spore-bearing bacillus which differs morphologically from the anthrax bacillus solely in showing rounded ends in cultures. It is motile. Its growth is more rapid, and it liquefies gelatin more energetically.

# SPORE-BEARING ANAEROBIC BACILLI IN WOUND INFECTIONS

The anaerobic bacilli which occurred with great frequency in wounds during the World War are common inhabitants of the intestinal tracts of man and other animals, and hence are frequently present in fertilized soils. The spores of most of these anaerobes develop in the intestine, and may remain viable for years in the soil. Cultures from the clothing of the men in the trenches almost always showed anaerobic organisms, especially the Welch bacillus and frequently tetanus bacilli.

Isolation of these organisms in pure culture is technically difficult, and much of the early work (prior to 1916) is untrustworthy, since many

of those investigating these organisms were dealing with mixed cultures. Repeated plating out of cultures is usually necessary, and Kendall regards the selection of a single spore by the Barber technique as essential for obtaining a pure culture.\*

These organisms do not thrive in living tissue. In order to produce infection there must be laceration with necrotic tissue or blood clot. War wounds contaminated with dirt and fragments of clothing, especially those produced by shell fragments, provide a suitable environment for their growth. Spreading occurs through injury to the surrounding tissues, both by the bacterial toxins and by mechanical pressure due to gas formation and oedema. Proteolytic organisms digest the dead tissue, producing blackening and the foul odor of gangrene, although they do not form toxins and cannot invade healthy tissue.

The important organisms found in these war wounds are divided into two broad groups according to their ability to ferment carbohydrates or digest proteins. This distinction is not clear cut, since some of the saccharolytic organisms have a slight proteolytic action as well, and vice versa.

The more important saccharolytic anaerobes which ferment carbohydrates vigorously with the production of acid and gas are:

Clostridium welchii

Clostridium oedematis-maligni (Vibrion septique)

Clostridium chauvei (Causes an analogous infection in animals only)

Clostridium oedematiens

 $Clostridium\ fallax$ 

Clostridium tertium

The more important proteolytic anaerobes which digest protein material with the formation of various amino-acids and sulphur compounds which blacken the cultures are:

 $Clostridium\ sporogenes$ 

Clostridium histolyticum

Clostridium welchii (Bacillus welchii) Welch, 1891.—This organism, commonly termed the "gas bacillus," was first isolated from gas gangrene cases and received the name Bacillus aerogenes capsulatus. Fraenkel gave the name B. phlegmonis emphysematosae to the same organism which he also obtained from similar lesions. The name B. perfringens is used by the French. This is the cause of foamy liver, and during the World War it was the most common cause of gas gangrene (75%), although nearly always associated with other organisms, especially Cl. sporogenes and

<sup>\*</sup>Methods for obtaining anaerobiosis are described on p. 829.

certain aerobes. Apparently only certain very virulent strains of the gas bacillus can produce gas gangrene when present alone and then only when

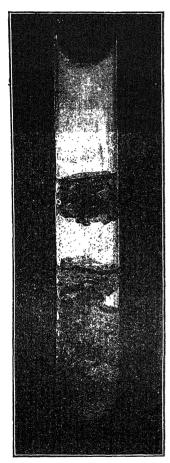


Fig. 8.—Cl. welchii agar culture showing gas formation. (MacNeal.)

much necrotized tissue is present in the wound. It is this requirement which led to the development of the surgical procedure of "debridement"

Morphology and cultural characteristics.—The gas bacillus is a large  $(5 \times 1.3\mu)$ , non-motile, Grampositive anacrobe with square-cut ends. It forms oval, centrally placed spores, which but slightly distend the rod. These spores are not formed in the wound, and it is difficult to bring about their development in ordinary culture media. Protein media, such as blood serum or egg, are the most favorable. It is inhibited by the presence of sugar in media. The bacillus shows a capsule in smears from animal tissues or fluids. Some strains exhibit haemolytic power when grown on blood agar. Cooked meat media are in general best for culturing, but the most characteristic cultural peculiarity is seen when the organism is grown in litmus milk, under anaerobic conditions. There occurs coagulation with disruption of portions of the coagulum into shreds, colored pink by acid formation and plastered against the sides of the tube. The gasriddled mass of coagulum remaining floats in a clear whey which has the odor of butyric acid. All the common sugars are fermented with the production of a large amount of gas. Glucose agar to which a little blood is added makes a very favorable medium. Coagulated serum is not liquefied, and indol is not formed in broth. The optimum temperature for growth is 38°C.

Laboratory diagnosis.—The presence of the Welch bacillus can be demonstrated by inoculating material into a tube of litmus milk, heating it to 80°C. for an hour, and incubating it anaerobically for 12 to 18 hours. If this organism is present the so-called "stormy fermentation" reaction described above may be seen. It is not produced by other anaerobes.

To obtain pure cultures, inoculate 3 or 4 cc. of the whey into the ear vein of a rabbit. After 5 minutes kill the rabbit and place the carcass in the incubator for 6 to 8 hours.

The body should become distended with gas and the organism should be obtainable from the foamy liver or the heart blood (Welch-Nuttall test).

At the American Ambulance a sterile cotton throat applicator is used to obtain the discharge from a wound. Each swab is sent to the laboratory in a tube. A glucose agar tube is boiled for ten minutes, then quickly cooled to  $42^{\circ}$ C. The swab is then introduced into the melted agar and well rubbed up in it. This agar is then quickly solidified in ice water and put in the incubator. For quick diagnosis the swab, coated with agar, is transferred to the tube from which it was taken and this latter is placed in a larger tube containing pyrogallic acid and sodium hydroxide to obtain anaerobiosis. In four or five hours a smear can be made from this swab and stained by Gram to note presence of the gas bacillus.

Toxin production.—Bull and Pritchett have produced a soluble toxin which causes both tissue necrosis and haemolysis. They found that all strains produced the toxin, but that the amount depended upon the virulence of the strain. Virulence could be increased by animal passage. By the injection of animals with this toxin they have produced an antitoxin. This antitoxin gives complete protection in animals, and has given favorable results in cases of gas gangrene in man.

Occurrence.—The gas bacillus is common in fertilized soils and in the faeces of man and other animals. Cultures from the clothing of men in the trenches almost always showed the Welch bacillus and less frequently the tetanus bacillus. Streptococci also were rather frequent. When a shell wound occurs we almost invariably have a gas bacillus infection, which during the first few days gives rise to a foul-smelling reddishbrown discharge. Smears from gas gangrene wounds, showing such discharge, have chiefly the gas bacillus and streptococci. In the second week the pus becomes more purulent and the gas bacillus is infrequent, while streptococci, staphylococci and coliform bacilli are abundant. The mechanical effect of pressure produced is the most important part of the infection, causing death of tissues from pressure on blood vessels. It also causes fragmentation of muscle tissue and scattering of the infection. In treatment provision must be made for escape of gas. Chlorinated solutions seem to be more efficient against the infection than the formerly recommended hydrogen dioxide.

The gas bacillus may also cause uterine infections, particularly those resulting from abortions. It may invade the blood immediately before death, and give rise to the gas bubbles in the organs and tissues which are sometimes seen at autopsies.

Kendall has called attention to the importance of this organism in a certain proportion of cases of summer diarrhoea of infants. (See chapter on faeces.)

Clostridium oedematis-maligni (Vibrion septique) Pasteur, 1877.—This organism was isolated by Pasteur from an animal supposed to have died of anthrax. Later, Koch isolated an anaerobe from garden soil which he named B. oedematis maligni although he regarded it as identical with Pasteur's Vibrion. He, however, reported it as liquefying gelatin, and, since the Vibrion septique has no such action, it seems certain that he was not dealing with that organism. Koch's bacillus is now considered to be identical with B. sporogenes.

Morphology and cultural characteristics.—Vibrion septique is a rather narrow bacillus with rounded ends measuring on the average  $6 \times 0.4\mu$ . It is motile, and, in a fresh

specimen of the blood of a guinea pig dying of the infection, appears in long undulating chains which move among the blood cells as serpents in the grass (Pasteur). In wet preparations from the liver of the guinea pig, these serpent-like forms are common. The bacillus forms oval spores which usually lie between the center and the end, but rather characteristic is the formation of a spindle-shaped organism with a large central spore and shrunken ends (lemon shape). Sporulation does not seem to take place in the body of the host. The organism is Gram-positive. It ferments milk with gas production, but less actively than does the Welch bacillus. It has little proteolytic action, not liquefying but only softening gelatin, and not acting on blood serum. It ferments glucose and lactose, but not saccharose. A similar anaerobe, B. chauvei, ferments saccharose. The colonies are filamentous and spreading. The odor of culture is sour or rancid—not foul.

Pathogenicity.—The organism was occasionally found in war wounds, usually in association with other anaerobes. It produces a soluble toxin which causes oedema and necrosis of tissues and contains an haemolysin. This toxin, like that of the gas bacillus, seems not to require a period of incubation, but excites the formation of an antitoxin. A rather large dose is required to kill an animal. The lesions of animals dying of the infection resemble those produced by the gas bacillus, but the Vibrion may be distinguished from the gas bacillus by its motility. In man the infection has been noted only in war wounds, but in herbivorous animals it may occur with or without wounds. Vibrion septique has been isolated from milk.

Clostridium chauvei (Bacillus chauvei).—This is an anaerobic spore bearer, called the bacillus of symptomatic anthrax, blackleg or quarterevil, which causes a rapidly developing emphysematous swelling, with a dark color, of the thighs. It has bulging, slightly oval spores at one end, but they are not distinctly terminal as are tetanus spores. It affects sheep and cattle but not man. It is a soil organism like those of tetanus and gas gangrene.

It is difficult to separate this organism from Vibrion septique, but as the latter alone is concerned in human infections this difficulty is important only for veterinarians. Robertson gives as differentiating points the fermentation of salicin but not saccharose by Vibrion septique while Cl. chauvei ferments saccharose but not salicin. Cl. chauvei does not exhibit the gliding-serpent chains which are such a feature of fresh liver emulsion and blood preparations from guinea pigs infected with Vibrion septique. Many have reported Cl. chauvei as Gram-negative.

Clostridium oedematiens (Bacillus oedematiens).—This bacillus is quite large  $(6 \times 1\mu)$  and is usually stated to be non-motile, although some authorities report young cultures as motile. It is more strictly anaerobic than the other pathogenic anaerobes. It liquefies gelatin. The spores, which (orm readily, are large  $(1.5\mu)$  and located excentrically. It produces a soluble toxin against which an antitoxin has been produced. Injection of the toxin (0.05 cc.) kills guinea pigs in one or two days.

This organism was a rather common one in the war wounds. Injected into the muscular tissues of a guinea pig it produces a gelatinous necrosis with only slight gas formation.

Clostridium fallax (Bacillus fallax).—This bacillus was a rather rare anaerobe of war wounds. It is smaller than the gas bacillus and is only slightly motile.

It produces a slight amount of gas but does not liquefy gelatin. Its spores, which are central or excentric, are not readily formed in culture media.

It produces a soluble toxin which gives rise to oedema. It is not a very pathogenic organism.

Clostridium tertium (Bacillus tertius).—This is a bacillus with rounded ends, averaging 5 by  $0.5\mu$  in size. It is sluggishly motile. The spores are strictly terminal. It does not liquefy gelatin and does not digest blood serum. It coagulates milk with slight gas formation. It has no pathogenic effect on laboratory animals but may cause gas formation in wounds.

Proteolytic Anaerobes of War Wounds. Clostridium sporogenes (Bacillus sporogenes).—This organism differs from the gas bacillus and the Vibrion septique in being actively proteolytic whereas they act on carbohydrates rather than on proteins. Next to the gas bacillus, it was the organism most frequently encountered in war wounds and was regarded as the main cause of their foul odor. Like the gas bacillus, it is often found in human or animal faeces and in fertilized soils.

It is a bacillus with rounded ends  $(5 \times 0.8\mu)$ , actively motile and Gram-positive; it liquefies gelatin and digests blood serum. It does not seem to be pathogenic but appears to exalt the virulence of the gas bacillus. It does not produce an exotoxin.

It is a common contaminant of other anaerobic cultures, and since its spores have great resistant power it is hard to separate it from organisms we desire to get in pure culture. Kendall considers Barber's single cell technique as valuable in effecting separation. Metchnikoff's organism, the Reading bacillus, Bacillus XI and—by some—Koch's organism of malignant ocdema are regarded as identical with Cl. sporogenes.

Clostridium histolyticum (Bacillus histolyticus).—This organism, like Cl. sporogenes, has marked proteolytic power, so that the intramuscular injection of cultures into guinea pigs may bring about digestion of the muscles down to the bone within 15 hours—without, however, having much effect on the health of the animal.

It is a motile bacillus, about 4 by  $0.6\mu$ , and forms oval spores which tend to be excentric. Cooked-meat media as well as blood serum are rapidly digested. No toxin is produced.

Therapy of anacrobic infections of war wounds.—If serum therapy is to be used successfully in cases of gas gangrene it is necessary to determine speedily and precisely which of these organisms are present, since different antitoxic sera are required for each. Merely to demonstrate the presence of Cl. welchii is not sufficient, since many cases show mixed infections with two or more anaerobes. Henry (1917) has suggested a method for a quick identification of the important saccharolytic anaerobes in wounds, Cl. welchii, Cl. oedematis maligni, and Cl. oedematiens. The material is inoculated into a cooked meat medium, and from this into a tube of milk. If the stormy fermentation occurs Cl. welchii is present. At the same time some of the culture is inoculated into two guinea pigs, one of which has received Cl. oedematis maligni and Cl. welchii antitoxin, and the other of which has received Cl. oedematiens and Cl. welchii antitoxin. If the first pig dies it ndicates that some organism other than Cl. oedematis maligni and Cl. welchii is present, and this is most often the Cl. oedematiens. This assumption is confirmed if the second pig survives. If the second pig alone dies one may draw the same conclusions with respect to the Cl. oedematis maligni. If both pigs die either both of these organisms or

some other anaerobes are probably present, and identification must be made by cultural methods.

Local treatment of lacerated wounds consists in immediate "debridement" in order to make conditions unfavorable for the growth of bacteria, in particular these anaerobes. Before closure is attempted smears from the wound should show few, if any anaerobes, and the presence of haemolytic streptococci should be excluded by cultures on blood agar.

#### **Botulism**

Clostridium botulinum (B. botulinus) Van Ermengem, 1896.—This organism produces botulism, a form of food poisoning. It is a spore-bearing anaerobe, and must not be confused with the S. enteriditis of Gaertner, a non-sporing aerobic bacillus also associated with meat poison-



Fig. 9.—Bacillus of botulism. (Kolle and Wassermann.)

ing. The organism is found in virgin as well as in cultivated soil, in manure, and on vegetables, fruits, etc. Burke believes that it may be disseminated by insects.

Morphology and cultural characteristics.—Cl. botulinum is a large, coarse bacillus 5 to  $7\mu$  by  $1\mu$ , occurring singly or in very short chains. It is slightly motile and stains by Gram. When sporulating the spore is near the end. It grows anaerobically on the ordinary media. It does not coagulate milk, which is a favorable medium. It ferments glucose with the production of a great deal of gas. Gelatin is usually liquefied. Cultures have a sour, rancid odor. It grows well at room tempera-

ture, less well at body temperature.

Toxin.—In contaminated foods and in cultures a powerful exotoxin is formed which is responsible for the symptoms produced. It is so potent that as little as 0.000001 cc. may kill a guinea pig. This toxin is destroyed easily by heating to  $80^{\circ}$ C. for  $\frac{1}{2}$  hour, or in a shorter time by boiling, unlike the sporulating bacillus which may survive boiling for some hours.

Two types of *Cl. botulinum*, A and B, are recognized in this country. Culturally they are similar, but their toxins differ, and the antitoxin of one type fails to neutralize the toxin of the other type. Type A. toxin produces a paralysis in chickens with weakness of the neck (the so-called limber neck), while type B. toxin produces no symptoms in them. A third type, C., occurs in Europe, but has not been found to be responsible for cases in this country.

Source of the infection.—In Europe the chief sources of infection have been contaminated meat, especially ham and sausage. In this country the outbreaks reported have resulted from eating canned vegetables, fruits, ripe olives and cheese. Weinzirl believes that commercial canned products are more likely to be safe than food preserved in the household. It is important to remember that the canned foods may not appear decomposed in any way, and yet may contain the bacilli and their toxin. The meat

BOTULISM 65

becomes infected only after the animal has been slaughtered, while in Gaertner meat poisoning the meat is from a sick animal, and is already infected at the time of slaughter. Since the toxin is destroyed by moderate heating, cooking food immediately before eating removes all danger of botulism.

Infection in man.—Clinical symptoms in man are due to the absorption of the toxin, which, unlike those of diphtheria and tetanus, is elaborated outside of the body and ingested preformed in the food. It is not destroyed in the alimentary tract, but is absorbed directly from the stomach and duodenum. The specific manifestations do not appear for from 12 to 24 hours or longer, although gastrointestinal disturbances may occur earlier. The characteristic symptoms are oculomotor paralyses with diplopia and mydriasis, and paralysis of the pharyngeal muscles with difficulty in swallowing. In fatal cases there are cardiac and respiratory symptoms from involvement of the medullary centers. no fever, and the mentality remains clear throughout. Although Cl. botulinum may be found in the gastrointestinal tract, and in fatal cases in the spleen, there is no danger of infection from one individual to another. as in the case of food poisoning due to Gaertner or paratyphoid infection. The toxin is ordinarily swallowed, and not elaborated in the gastrointestinal tract.

Examination of food.—To ascertain the presence of the toxin in the food, filtered extracts of the suspected food may be injected intraperitoneally into a guinea pig, and characteristic bulbar and pupillary symptoms will result, with death by cardiac or respiratory failure. Another animal should be injected together with the antitoxin as a control. Cultures can be made by heating an emulsion of the food at 60°C. for an hour to destroy non-sporing bacteria, and making anaerobic shake cultures in glucose agar. These should be incubated anaerobically at room temperature in the dark. Filtrates from broth cultures can be tested for the presence of the toxin by guinea pig inoculation. Feeding chickens with suspected material has been used to differentiate the types.

Serum treatment.—An antitoxin which is said to have therapeutic value in botulism has been prepared by Kempner, and by Dickson and Howitt, by the immunization of goats. A polyvalent serum containing antitoxin for both types must be used. Since the serum does not seem to be of much value when the symptoms have fully developed, it should be used early. Without serum treatment death occurs in about 70% of the cases, and may take place within 48 hours.

#### Tetanus

Clostridium tetani (B. tetani) (Carlo and Rattone caused tetanus in rabbits by inoculating pus from a human case, 1884; Nicolaier produced

tetanus by injecting animals with garden soil, 1885; Kitasato obtained pure cultures by incubating tetanus-containing material for 48 hours, then heating at 80°C. for one hour, thus killing non-sporing organisms, culturing anaerobically and producing tetanus in animals by inoculating the culture, 1885).—The tetanus bacillus is common in the faeces of horses and cattle, hence its frequency in cultivated soils. The horse is the most susceptible animal, next the guinea pig, then the mouse. The infection occurs in cattle and sheep. Dogs are much less susceptible. Fowls are practically immune.

Morphology.—It is a long slender bacillus  $(4 \times 0.4\mu)$ , slightly motile and forming a large  $(1.3\mu)$  drumstick spore. Certain other anaerobes, as Cl. tertius, etc. form similar

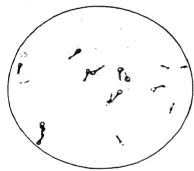


Fig. 10.—Tetanus bacilli showing end spores. (Kolle and Wassermann.)

terminal spores so that a diagnosis of tetanus cannot be made by this finding alone. It is Gram-positive, but degenerating organisms in old cultures may lose this characteristic.

Cultural characteristics.—The tetanus bacillus grows anaerobically on ordinary media. The addition of glucose or fresh sterile tissue to the culture facilitates its growth, especially in the absence of complete anaerobiosis. Colonies on agar plates are small, and are surrounded by a network of matted filaments. In stab cultures in glucose agar it shows as an inverted pine-tree growth. It liquefies gelatin very slowly. Milk is not coagulated. None of the carbohydrates are fermented. Cultures have a slight odor of putrefaction.

Viability.—The spores may remain viable in the soil for years. They are very resistant to heat and ordinary antiseptics. They may withstand boiling for an hour or longer, but are usually killed within 5 minutes in the autoclave.

Variation.—Motile and non-motile variants, and smooth and rough colony strains have been described.

Types.—Tulloch (1917) has differentiated three main types of tetanus bacilli by agglutination and agglutinian absorption tests. However, there does not seem to be any type specificity in the toxin, so that an antitoxin made from type 1 will protect against the toxins of the other types. More recent work has differentiated 5 further groups (Fildes, 1929).

Toxin.—The tetanus bacillus produces a soluble toxin. It is one of the most powerful poisons known and it is estimated that 0.0002 Gm. is fatal for man. It is said to be 20 times as poisonous as dried cobra venom. It can be prepared by growing the bacilli anaerobically in broth, and filtering after 10 to 14 days. There are, in fact, two toxins; tetanos pasmin which damages the tissues of the nervous system, and tetanolysin, of minor importance, which causes haemolysis of red blood cells. That the disease is due to the toxin is shown not only by the experimental production of tetanus by means of toxic

## SYMPTOMS OF TETANUS

filtrates, but also by the fact that, if spores are carefully freed of all toxin by washing, and then introduced, they do not cause tetanus, but are phagocyted. When the toxin is injected into susceptible animals death occurs in 12 to 24 hours with typical symptoms of tetanus.

The route by which the toxin reaches the nervous system has been the subject of extensive investigation. It has been believed that the toxin produced locally by the growth of the bacilli was absorbed by the local motor end plates and travelled directly to the central nervous system by way of the axis cylinders. However, Abel (1934) has published evidence to show that the toxin reaches the central nervous system by way of the blood stream.

The disease in man.—Tetanus is caused by the action on the central nervous system of a toxin produced by the growth of tetanus bacilli in a deep, penetrating wound. Such wounds, especially when there is much tissue destruction, afford a suitable anaerobic environment. The presence of other bacteria in the wound favors the growth of tetanus bacilli, especially the presence of ('l. welchii, which is frequently associated, and which creates a favorable soil by causing extensive tissue destruction. The spores germinate readily only in an oxygen tension which is lower than that in normal tissues. Tetanus has occurred following childbirth or abortions, and in the new born from infection of the umbilicus. It has occurred after surgical operations from insufficiently sterilized catgut or other supplies, and following vaccination and hypodermic injections.

After infection of a wound there is a definite incubation period before the first symptoms develop—usually from 5 to 10 days in the acute cases, although it may be much longer. As a rule, the shorter the incubation period, the more severe is the disease. The muscle spasms develop first, as a rule, in the neck and jaw muscles and the muscles around the mouth, giving the characteristic "risus sardonicus." Later the muscles of the trunk and back become affected. The muscle spasms are associated with violent pain, dyspnoea, and difficulty in swallowing.

In tetanus, as in diphtheria, the bacilli remain localized in the lesion, and the injury to other organs is caused by the toxin which is absorbed from the wound. However, spores have been found in the regional lymph glands, and also in the liver and spleen in fatal cases.

The term "chronic tetanus" has been applied to atypical cases developing slowly, with a relatively mild course. Cases may occur several months after injury or operation, especially with low grade bone infections.

Bacteriological examination of a wound.—Tetanus bacilli can rarely be demonstrated in films made directly from the wound. Animal inoculation is more dependable than cultural methods.

The wound should be curetted and some of the tissue fragments inserted into a pocket in the subcutaneous tissue of the thigh of a guinea pig. The remainder may be inoculated into glucose agar stabs or directly on Löffler's blood serum. On this medium the growth of contaminating organisms enables the tetanus bacilli to multiply aerobically. In these cultures the development of a foul, sour odor is suggestive. Films from such cultures frequently show the drum-stick spores. If these are found, heat an emulsion of the growth to 80°C. for ½ hour to kill non-sporing bacteria, and inoculate a deep glucose agar tube, and cultivate anaerobically.

A more rapid method is to seal the material obtained in a capillary pipette, and heat to 80°C. for 15 minutes. This can then be plunged into a deep tube of glucose agar which is inoculated along the stab. The tube can be covered with sterile liquid petrolatum and incubated. However, better anaerobiosis can be obtained by the Buchner or Wright method.

The filtrate from cultures, even when mixed, can be inoculated into animals (rats or mice) to demonstrate the presence of the toxin. This is the most reliable method for demonstrating the presence of tetanus bacilli. A control animal inoculated with the filtrate together with antitetanic serum should be protected.

Antitoxin.—The antitoxin is produced by injecting horses with increasing doses of tetanus toxin, at first adding sufficient antitoxin to neutralize it. A high degree of immunity to the toxin is developed. The method of standardization established by law in the U. S. is based on the work of Rosenau and Anderson at the U. S. Hygienic Laboratories. The antitoxin unit is defined as 10 times the minimal amount of serum necessary to protect a 350 gram guinea pig for 96 hours from 100 M.L.D. of a standard toxin. Standard antitoxin can be obtained from the National Institute of Health, by means of which others can determine the strength of their own toxin, and indirectly of their antitoxin. This unit has relatively 10 times the potency of the unit of diphtheria antitoxin.

Preventive measures. Passive immunization.—The practical value of tetanus antitoxin is largely limited to its use as a prophylactic measure. Its effectiveness for this purpose, if properly used, has been demonstrated conclusively. It should be given immediately in doses of 1500 units, after any deep injury which may be contaminated by soil or dirt, especially if there is laceration of tissue. As the immunity from a single injection lasts only about 10 days, a second dose will often be required at that time if the wound has not healed. It is particularly important to repeat the injection before subsequent operations on the infected area, since otherwise such procedures may start up an acute tetanus. Anaphylactic reactions can be obviated by a preliminary desensitizing dose. (See section on Hypersensitiveness.)

Active immunization.—By incubating tetanus toxin with 0.3% formaldehyde for several months, its poisonous properties can be destroyed without affecting the antigenic powers. With such tetanus toxoid, or anatoxin, it has been possible to immunize animals actively to tetanus, and to stimulate the production of substantial amounts of antitoxin in man. Bergey (1934) found that alum-precipitated toxoid was more antigenic and gave no local or general reaction. He recommends one injection followed by a

second after three months. A third injection is given on receiving an injury. It is possible that this method of active immunization may prove of practical value in armies, and in occupations in which the risk of tetanus is great.

Scrum treatment after symptoms have developed is far less efficacious than that of diphtheria, largely because the toxin has already become fixed to the nerve tissues. The nerve cells have a greater affinity for the toxin than has the antitoxin, and when once injured do not recover as readily as other body cells. The mortality of untreated cases developing within 10 days is over 80%. Serum treatment has little effect in cases with an incubation period of less than 5 days, but it has been reported to lessen the mortality substantially in those cases with incubation periods of 5 to 10 days or longer (to 30% according to Courtois-Suffit and Giroux (1918) and to 52% according to Wainwright, (1926)). Simple subcutaneous injections are useless. It must be given intravenously, repeatedly, in doses of 20,000 units. Some advise a total of up to 100,000 units the first day. It is customary also to give a dose subdurally at the onset, (Park and Nicoll, 1914), although some others regard this as useless and even dangerous. It is also essential that the wound be excised or thoroughly cleaned of all dirt and necrotic tissue and that the spasms be controlled by powerful sedatives like avertin (Teichmann, 1932).

## CHAPTER IV

# STUDY AND IDENTIFICATION OF BACTERIA—MYCOBACTERIA, CORYNEBACTERIA AND ACTINOBACILLUS. KEY AND NOTES

Key for Bacilli.—Having branching characteristics; showing parallelism, branching, curving forms, V-shapes, clubbing at ends, segmental staining, etc.

Acid-fast.—Mycobacterium. {Cultures more or less wrinkled and dry. {More like moulds.

- I. Grow rapidly on ordinary media at room temperature.
  - (1) Mycobacterium phlei. (Timothy grass bacillus of Moeller.)
  - (2) Mycobacterium butyricum. (Butter bacilli as reported by (a) Rabinowitsch and (b) Petri.)
- II. Grow only at body temperature. Scanty growth or none at all on ordinary media. Media of preference are: (a) Solidified blood serum, (b) glycerin agar, (c) glycerin potato and (d) egg media.
  - A. Cultures fairly moist, luxuriant, and flat. Opt. temp. 43°C.
    - (1) Mycobacterium avium. (Bacillus of avian tuberculosis.)
  - B. (a) Cultures scanty, wrinkled and dry. Appear in ten to fourteen days. Opt. temp. 38°C. Bacilli longer (2.5μ), narrower, more curved, more regular in shape and staining than are bovine; vacuolation more marked. Smear from organs of inoculated guinea pig shows few bacilli. Less virulent for rabbits.
    - (1) Mycobacterium tuberculosis (hominis). (Bacillus of human tuberculosis.)
    - (b) Cultures as above, but even more scanty. Bacilli shorter  $(\tau.5\mu)$ , thicker, less vacuolated. Smear from organs of guinea pig shows many bacilli.
      - (1) Mycobacterium tuberculosis (bovis). (Bovine tubercle bacilli.)
  - C. Very difficult to cultivate (Czaplewski).
    - (r) Mycobacterium smegmatis. (Smegma bacilli for various animals.)
- III. Are not cultivable by ordinary methods.
  - Mycobacterium leprae. (B. leprae.) Found chiefly in nasal mucus and in juice from lepra tubercles. Less often in nerve leprosy.
  - (2) Mycobacterium leprae murium. (Bacillus of rat leprosy.) Indistinguishable from *M. leprae* except by inoculation into young rats of the same species.

 $\label{eq:Nonacid-fast.-Corynebacterium and Actinobacillus. Colonies more flat and moist. \\ Like other bacteria.$ 

- I. Gram-positive: Corynebacterium.
  - (1) Corynebacterium pseudodiphthericum. (B. hoffmanni.) Very luxuriant growth on ordinary media. Colonies often yellow to brownish. Short, thick and stain uniformly.

- (2) Corynebacterium diphtheriae. Moderate growth on ordinary media. Best media are blood scrum (Löffler's) or blood agar. Has metachromatic granules at poles.
- (3) Corynebacterium xerose. Scanty and slow growth on nutrient media.
- II. Gram-negative: Actinobacillus.
  - (1) Slender, poorly staining rods, forming threads with a tendency toward branching. Do not ferment carbohydrates. Characteristic honey-like growth on potato. Actinobacillus mallei (Glanders bacillus). Cause of glanders, affecting horses, man, sheep, and goats.
- Note: A. pseudomallei (B. whitmori), the cause of melioidosis, a glanders-like disease in man and rodents occurring in Burmah and the Malay States, is serologically almost identical with A. mallei, but differs from it in its motility and the character of its growth on laboratory media.

# THE GROUP OF ACID-FAST BRANCHING BACILLI

There is a large number of non-pathogenic acid-fast bacilli which are important only because they may be confused with the true tubercle bacillus. Their colonies correspond more or less with different types of tubercle bacillus colonies, being either dry and wrinkled like human, or moist and irregularly flat as avian. Eventually the moist colonies become dry and wrinkled. They have been isolated from butter and milk; from grasses, especially in timothy grass infusion; from various excretions of animals as dung, urine, etc., and from the skin, nasal mucus, cerumen and tonsillar exudate of man, where they occur normally.

These non-pathogens differ from the tubercle bacillus in five important essentials:

- 1. Grow readily on any media.
- 2. Show more or less abundant growth, or colonies, in twenty-four hours.
- 3. Have no pathogenic power for guinea pigs when inoculated subcutaneously.
- 4. Do not require body temperature for development, but grow at room temperature or a little above.
  - 5. Are morphologically shorter and thicker.

Many of these organisms if injected intraperitoneally into guinea pigs will produce a peritonitis with false membrane. Some also produce granulation tissue nodules which may be confused with true tubercles. When injected intravenously localization is renal—very rarely pulmonary. Lesions tend to suppurate rather than to caseate.

Mycobacterium smegmatis (Bacillus smegmatis).—This acid-fast organism is chiefly important from the fact that such bacilli may be found in urine and be reported as tubercle bacilli.

They show a greater tendency to appear in clumps, like lepra bacilli, but may have the appearance of typical tubercle bacilli. It is usually stated that they decolorize easily in alcohol, but I have found smegma organisms in urine as resistant to acid alcohol as tubercle bacilli. Such organisms are found in preputial and vulvar secretions, and such location accounts for their presence in urine. The Lustgarten bacillus, reported in 1884 as the cause of syphilis, was probably a smegma bacillus. It is very difficult to obtain cultures (blood or serum media are necessary). For differentiation always inoculate a guinea pig, for which, as for man, the organism is non-pathogenic.

#### STUDY AND IDENTIFICATION OF BACTERIA

#### Tuberculosis

Mycobacterium tuberculosis (hominis) (Bacillus tuberculosis) Koch, 1882.

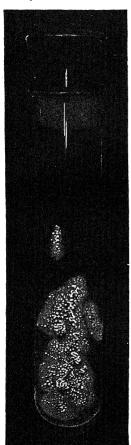


FIG. 11.—Mycobacterium tuberculosis; glycerin agar-agar culture, several months old. (Curtis.)

Morphology.—This is a rather long, slender rod,  $2-5 \times 0.3\mu$ , straight or slightly curved, with rounded ends. It is found singly or in clumps, in which the bacilli tend to lie parallel or form an acute angle with each other. Many of the organisms show a characteristic beaded appearance in stained films. In old cultures filamentous forms occur, and true branching has been observed. It is non-motile, and Gram-positive.

Staining.—Tubercle bacilli do not stain easily with the ordinary dyes owing to the waxy material in the organism. and when once stained, are not decolorized by the acid alcohol used in the ordinary staining methods. Ziehl-Neilson method is the one most commonly used. Pappenheim's stain is used chiefly for the differentiation of the tubercle bacillus from the smegma bacillus. In young cultures many of the rods may be non-acid-fast. Much has described non-acid-fast, Gram-positive granules in cultures and in tuberculous lesions which he believes are resistant forms. It is claimed that material containing these granules, but without demonstrable acid-fast forms, will cause tuberculosis in animals with acid-fast bacilli in the tissues. A filtrable form has also been described, and it has been suggested that these types represent different phases in the life cycle of the tubercle bacillus. A combination of the acid-fast and Gram staining methods. recommended by Fontes (see § on staining methods), is very satisfactory for demonstrating the Gram-positive rods and granules, and the acid-fast forms.

Cultures.-Primary cultures should be made on a mixture of egg volk and glycerin agar or on blood media. Petroff's or Dorsett's egg media are recommended. For subcultures either glycerin agar, glycerin potato or glycerin broth may be used. Growth on all media is slow, and colonies become visible only after one or two weeks, and gradually cover the surface with an irregular, crinkled, dry, whitish to buff-colored growth. In fluid media such as glycerin broth a similar growth spreads out over the surface forming a pellicle. In inoculating solid media with tissue, the material should be rubbed thoroughly over the medium. Transplants to fluid media should be made by floating a flake of the culture on the surface. Tubercle bacilli require abundant oxygen for growth. The temperature for growth must be between 30°C. and 42°C. (optimum 37°C.).

Viability.—The tubercle bacillus is killed by heating at 60°C. On account of its waxy covering it is very resistant to drying and to antiseptics, especially in sputum. It

is very susceptible to direct sunlight. Cultures remain viable for months if kept moist.

Types of tubercle bacilli—human, bovine, avian; differentiation.

- I. The bovine type, belonging to the natural tuberculosis of cattle.
- II. The human type, the type usually found in man.
- III. The avian type, belonging to natural tuberculosis of fowls.

Bovine and human types.—Bacilli of the bovine type are shorter and thicker, and stain more solidly than the human type and tend to be irregularly arranged, whereas the human types are longer, slightly curved, and tend to lie parallel.

The bovine type grows slowly on serum, and at the end of two or three weeks shows only a thin grayish uniform growth which is not wrinkled and not pigmented. The human type grows more rapidly and tends to become wrinkled and pigmented. Subcutaneous inoculation of 50 mg. of bovine culture into the neck of calves produces generalized tuberculosis whereas a similar injection of human organisms produces only an encapsulated local lesion.

Intravenous injection of 0.01 to 0.1 mg. of bovine culture into rabbits causes general miliary tuberculosis and death within five weeks. With human cultures in doses of 0.1 to 1.0 mg., similarly injected, the majority of rabbits live for three months or more.

Subcutaneous injection of 10 mg. bovine tubercle bacilli causes death in 28 to 101 days. Similar injection of human organisms in doses up to 100 mg. do not kill rabbits after periods of from 94 to 725 days. The duration of life in injected guinea pigs is longer with human than with bovine inoculations.

Subcutaneous injection of bovine strains into cats produces generalized tuberculosis, whereas the cat is resistant to human strains thus given.

Avian type.—The avian type grows at 43°C. fairly luxuriantly, as a moist, more or less spreading culture. It grows much better on glycerinated agar than on serum. Morphologically it is like the human type, but shows less tendency to form compact masses. It is very pleomorphic.

Fowls may be infected by intravenous or subcutaneous injection, or as the result of feeding. After feeding, the lesions are chiefly of the alimentary tract; after injections, of spleen, liver and lungs. Avian cultures are more virulent for rabbits than human but less so than bovine. The mouse is the only mammal besides the rabbit in which avian tubercle bacilli can cause a generalized tuberculosis. It is believed that there is no danger to man from avian types.

Cold-blooded animal type.—Certain acid-fast organisms have been isolated from fish, frogs and snakes, and from algae. These organisms grow much more rapidly than the other types (three to four days), and grow best at 20° to 24°C., growth ceasing at 36°. The colonies are round and moist. As a rule they do not produce tuberculin and may be considered as harmless saprophytes. However, Aronson has reported a strain from salt-water fish which is tuberculin-producing and pathogenic for mice, pigeons and frogs, but non-pathogenic for guinea-pigs.

Variation.—Human, bovine and avian strains have been dissociated into R and intermediate colony forms. The S forms are more virulent than the R forms. Transformations from one type to another can be induced by cultivation on suitable media. Both S and R colonies have been found in cultures obtained directly from patients.

Incidence of human and bovine tuberculosis.—Tuberculosis is the most common of human diseases. The incidence of infection increases with age, but the mortality is greatest between the ages of 15 and 40. A great majority of adults show old tuberculous lesions at autopsy, and from 50 to 90% of children over 14 give a positive reaction to the tuberculin test.

Tuberculous infections of the skin, bone, and glands are frequently of the bovine type, whereas pulmonary and laryngeal lesions are almost always of the human type. Park and Krumwiede in a study of more than 1000 cases of all ages found about 10% due to bovine types. Of 686 adult cases only 1.3% were due to bovine strains, whereas of 352 cases under 16 approximately 25% were bovine infections. Of 592 cases of pulmonary tuberculosis in children and adults, not a single case could be regarded as bovine.

Atrium of infection.—In the bovine types of human tuberculosis the portal of entry is the alimentary tract. It is believed that the tubercle bacillus may penetrate the intact mucosa of the intestinal tract without causing pathological changes and reach other areas in the body through the lymphatics. In some infections due to human strains the organisms apparently enter the body in the same way from food or milk which has been contaminated directly from human cases. Most of the cases, however, result from the inhalation of infected dust or from droplet infection from careless patients with open lesions. The resistance of the bacilli to drying, especially in sputum, permits them to be disseminated by the fomites and by dust and dirt.

Whether or not active disease develops after their entry into the body depends upon their virulence, and upon the resistance of the body to their invasion. It is probable that ordinarily the actual infection occurs in childhood, and that the disease may become active later on, whenever the general resistance is lowered to such an extent that the bacilli are enabled to multiply and invade the tissues. Possibly, by direct contact with an infective individual one may receive suddenly a sufficiently great number of bacilli to overwhelm the natural resistance. The susceptibility of an individual varies markedly with age and is greatest in the first few years of life.

Many of the symptoms of pulmonary tuberculosis are due to secondary infections. Streptococci, pneumococci, staphylococci, or influenza bacilli are frequently present in the neighborhood of the tuberculous lesion and can be found in the sputum.

Laboratory Diagnosis.—This depends chiefly upon the demonstration of tubercle bacilli in the body fluids or tissues by means of stained smears,

cultures or guinea pig inoculation. The existence of an infection can also be proved by demonstrating hypersensitiveness to the proteins of the tubercle bacillus by means of tuberculin tests.

Slained smears.—The diagnosis may be made directly by acid-fast stains of infected material. In the case of sputum the finding of acid-fast, beaded bacilli is almost conclusive evidence of pulmonary tuberculosis. When, however, the urinary sediment from an uncatheterized specimen contains acid-fast bacilli, they must be distinguished from smegma bacilli. To avoid this confusion a catheterized specimen should be obtained and the result confirmed by guinea pig inoculation. Pleural and other exudates can be centrifuged and the sediments examined in this way. In tuberculous meningitis tubercle bacilli can often be demonstrated by staining the film which forms in the cerebrospinal fluid after standing a few hours.

Sometimes the number of bacilli is small in proportion to the volume of the material to be examined, and it is necessary to concentrate them by digestion of the specimen with some solvent such as antiformin, as described in the section on sputum.

Cultures can be made directly upon suitable media from material which is not contaminated. Cultures from the sediments of pleural or other exudates, or the film from a tuberculous spinal fluid may show tubercle bacilli when they are not demonstrable by smears or by guinea pig inoculation. When cultures are made from sputum or other contaminated material, other bacteria must be killed by treatment with alkali or acid. After this digestion the centrifugalized sediment is neutralized, and inoculated on Petroff's or other suitable medium. (Details of this method are given in the section on sputum cultures.) Pure cultures can often be obtained in this way. Clough has grown tubercle bacilli from the blood in cases of military tuberculosis by laking the red cells with sterile distilled water, and planting the sediment after centrifugalization on blood agar slants. The bacilli are rarely present in the blood in other forms of tuberculosis, however. Growth is slow and even under the most favorable conditions colonies do not become visible in less than a week. Within a lew days, however, the tubercle bacilli will have multiplied sufficiently to demonstrate them in smears from the culture.

Guinea pig inoculation.—The guinea pig is susceptible to both bovine and human tuberculosis, and is used to demonstrate the organisms when they cannot be found by other methods, and to distinguish between tubercle bacilli and non-pathogenic acid-fast bacilli.

The animal is injected subcutaneously in the groin. If the material is badly contaminated, it must be treated with acid or alkali, as in making cultures, or the animal may die from some secondary infection. If the tubercle bacillus is present, a local swelling develops which may cascate and ulcerate. Smears from the ulcerated area often show the bacilli. The regional glands become involved and later the mesenteric and other lymph nodes. The lesions may be looked for in from 4-6 weeks and death usually occurs in about 2 months. At autopsy the characteristic lesion is an enormous enlargement of the spleen, which is studded with grayish or yellowish tubercles. Smears and cultures from the spleen show the tubercle bacilli. The liver, lungs, and peritoneum are often involved. Block suggests that the lymphatic glands in the groin be damaged by squeezing the tissue between the fingers before inoculating the animal and claims that tubercle bacilli may be demonstrated in these damaged glands within 10 or 12 days. Infected guinea pigs become highly sensitive to tuberculin after 2 or 3 weeks, and will die acutely within 24 hours if given a subcutaneous injection of a large dose (1 or 2 cc.) of

O.T. at this time. This phenomenon is utilized to determine whether tuberculosis has developed from the inoculation without waiting for the appearance of gross lesions. It is desirable to inoculate a second animal as a control and allow it to live until lesions have developed. Intracutaneous tuberculin tests may be used also to determine whether an inoculated animal has become infected.

Tuberculin.—When killed tubercle bacilli or the by-products of their growth in culture media are injected into animals infected with tuberculosis, in quantities which are innocuous for normal animals, they cause an acute local inflammatory reaction at the site of inoculation, and often a violent general reaction. This is due to a hypersensitive (allergic) reaction to the specific protein of the tubercle bacillus (tuberculo-protein). or some of its decomposition products. The term "tuberculin" is applied to any one of numerous preparations which are capable of eliciting such a reaction. A positive tuberculin reaction is manifested in three ways. (1) A local reaction with erythema, infiltration, and even necrosis. (2) A general reaction with fever, prostration, and in severe instances sudden death. (3) A focal reaction, with increased activity of the tuberculous process. In pulmonary tuberculosis this may be manifested by increased cough, sputum or haemoptysis; or in joint tuberculosis by increased pain, swelling, redness, and disability of the joint. All of these phenomena are utilized in diagnosis, but it is obvious that a marked general or focal reaction should be avoided lest it cause serious injury to the individual.

Tuberculin Preparations.—Koch's "Old Tuberculin" (O.T.) is the form which has been most used for diagnostic purposes. It is made by concentrating the fluid from a 6 weeks-old culture in 5% glycerine broth to  $y_{10}$  its original volume by heating at 80°C. and filtering. Strains of tubercle bacilli must be chosen which yield active filtrates. Different lots vary considerably in activity, and no satisfactory method of standardization has been devised.

The purified protein derivative (P.P.D.) is a highly purified form of tuberculo-protein which is obtained by growing the bacilli in a synthetic medium free from peptone or foreign protein (Dorset's medium), removing the constituents of the culture filtrate by ultrafiltration, and precipitating the tuberculo-protein with trichloracetic acid. A product of uniform potency can be reproduced at will, regardless of the strain of bacillus used. It keeps indefinitely as a dry powder, or in concentrated solutions, but like all tuberculins deteriorates rapidly in high dilution. It is non-antigenic (a haptene), but elicits a skin reaction in a dose of about  $\cancel{1}_{100}$  that of the average O.T. It is now available in tablet form in 2 concentrations, each containing material for 10 tests. This preparation promises to replace all other tuberculins for diagnostic purposes. Its effectiveness for therapeutic use, however, has not been determined as yet. (See supplement, Amer. Rev. Tuberc., Dec. 1934.)

Among the many other tuberculins, used mainly for treatment, may be mentioned the T.R. and B.E. of Koch (ground up bacilli suspended in water or glycerin solutions without heating), and the bouillon filtrate of Denys (similar to O.T. but not heated or concentrated).

Modes of Application for Diagnosis.—Subcutaneous injection.—Koch's O.T. is used, the initial dose being usually 0.2 mg. If this gives no reaction, subsequent doses of 1.0 mg., and 5.0 mg. may be tried at 5 days intervals. A positive reaction is indicated by a rise in temperature of at least 1° within 24 hours, or by a definite focal reaction. A local reaction accompanies the general reaction, but alone is less significant. A positive reaction usually indicates active tuberculosis of clinical importance, but there is some risk of causing an exacerbation of the disease by its use. The test is applicable only to patients with a normal temperature, and necessitates remaining in bed for several days with regular temperature observations before and after each injection.

Ophthalmic lest (Calmette).—This test, made by instilling a drop of 0.5% purified O.T. into the conjunctiva, has been largely abandoned because of occasional injury to the eye.

Culaneous test of Von Pirquet.—A drop of undiluted O.T. is applied to the forearm, and the skin is lightly scarified through it. A control scarification is made through a drop of salt solution. A positive reaction appears within 24, or rarely 48 hours, as a small, indurated, red papule. Practically, the test is of value only in young children, since a large percentage of healthy adults give a positive reaction. It is less sensitive and precise than the following test.

Intracutaneous reaction of Mantoux.—This is carried out by injecting 0.1 cc. of a series of dilutions of O.T. intracutaneously into the skin of the forearm. A positive reaction is indicated by the appearance in 24 to 48 hours of a local erythema from 0.5 to several cm. in diameter, with a perceptible area of induration in the center. In marked reactions there may be a large area of induration with vesiculation or necrosis, and some general reaction. This method is the most sensitive, and is safe if given carefully, beginning with a high dilution. If a high degree of sensitiveness is suspected, or if it is essential to avoid a general reaction (as in inflammatory lesions of the eye), one should start with 0.1 cc. of a dilution of 1-1,000,000 of O.T. (0.0001 mg.), and, if negative after 24 hours, inject in the same way a dilution of 1-100,000. This is repeated daily using solutions 10 times as concentrated each time until a positive reaction is obtained, or until 0.1 cc. of a 1-10 dilution (10 mg.) has been given. In some cases one may give 2 or 3 of the higher dilutions at a time, or start with a 1-10,000 dilution. By giving a series of injections of graded dilutions in this way, it is possible to determine the degree of hypersensitiveness.

The P.P.D. may be used instead of O.T., giving first o.r cc. of the solution made by dissolving one of the weaker tablets in r cc. of the buffer phosphate solution furnished. This dose contains 0.0002 mg. of P.P.D. (roughly equivalent to 0.02 mg. O.T.). If this is negative, a second injection of 0.r cc. is given of a similarly prepared solution of the stronger tablet containing a dose of 0.05 mg., 250 times the first dose. In a survey reported by Long, Aronson and Seibert, no unpleasant reactions to the larger dose were observed in patients giving a negative reaction to the first. A few marked reactions, however, were observed after the first dose, and in cases in which marked hypersensitiveness is suspected it is conservative to start with a dose of  $\frac{1}{100}$  of that recommended (0.000002 mg.).

Significance of tuberculin reactions.—A positive reaction always means tuberculous infection, but not necessarily active disease. The practical

significance of a positive reaction cannot be determined merely from the degree of the reaction, but only from a complete study of the patient by an experienced clinician. There is no direct relationship between the degree of hypersensitiveness and the gravity of the disease. A negative reaction does not exclude tuberculosis. The reaction may be negative in individuals with latent, healed lesions, in cases with active, rapidly progressive lesions, and in miliary tuberculosis. It may be temporarily lessened or abolished by other acute infections, notably measles and influenza.

By therapeutic administration of tuberculin it is usually possible to lessen markedly the degree of hypersensitiveness, and in some types of the disease this appears to be beneficial. It is contraindicated in acute febrile cases. Its use is largely limited to relatively inactive cases with localized lesions, such as bone or gland tuberculosis, and to inflammatory lesions of the eye. No one type of tuberculin has been shown to be superior to the others. The best results appear to be obtained by small doses which cause no reaction. The initial dose should not usually exceed  $^{1}_{160}$  of the minimal skin-reacting dose, and increases should be made very gradually.

Active immunization.—Although some degree of immunity can be produced in animals by the injection of dead tubercle bacilli, the protection so conferred is usually slight. On the other hand, a guinea pig with an active tuberculous lesion containing living organisms of low virulence is usually resistant to superinfection, even if a highly virulent strain is injected. Calmette has attempted to produce a similar resistance in man by feeding children with living cultures of an attenuated strain (B.C.G.) which, although producing tuberculin, has lost its pathogenicity by prolonged cultivation. The efficacy of this procedure can be judged only after prolonged trial. In this as in all vaccination with living organisms there is always a potential danger of a spontaneous restoration of virulence, and many investigators are opposed to this procedure on that account. Petroff and Steenken (1930) claim to have produced virulent, smooth forms from 4 different cultures of Calmette's B.C.G. On the other hand, many thousands of children have been treated in this way with practically no ill-effects which could be ascribed to the vaccine.

## LEPROSY

Mycobacterium leprae (Bacillus leprae) Hansen, 1874.—This organism is the accepted cause of human leprosy and is usually present in great profusion within characteristic cells in the specific lesions.

Morphology.—In size, shape and staining reactions M. leprae is practically indistinguishable from the tubercle bacillus, but can be differentiated by the following points.

(1) Leprosy bacilli are found ordinarily in huge numbers in the lesions, chiefly within the so-called lepra cells, and are often grouped in packets like a bundle of cigars tied together, or arranged in a palisade. Chains are never seen. (2) They usually stain more solidly, and the granules are coarser and more widely spaced. They may be stained by the ordinary strong bacterial stains, such as cold dilute carbol-fuchsin, and by the Gram stain with which it is Gram-positive. (3) They do not resist the decolori-

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zation in acid-fast staining quite so well, although there is some variation in the individual strains in this respect. In the Ziehl-Neelsen method with 3% HCl in alcohol they decolorize much faster than the tubercle bacillus, while with 20% H<sub>2</sub>SO<sub>4</sub> in water they may hold their color almost as well. Some prefer to use a 5% solution of the latter. (4) They do not grow in cultures or produce disease in animals, and they may be differentiated with certainty from the tubercle bacillus by their failure to cause lesions in inoculated guinea pigs.

Cultivation of the leprosy bacillus has been attempted repeatedly, with, for the most part, negative results. Some investigators, however, have obtained growths of acid-fast bacilli (both chromogenic and non-chromogenic) or of acid-sensitive diphtheroids which in some instances developed acid-fast forms upon cultivation. Branching bacilli, and granular and coccoid forms have also been described. Recently several investiga-

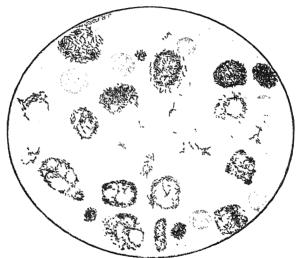


Fig. 12.—Section of spleen showing lepra cells and lepra bacilli. X800. (By permission from Manson's "Tropical Diseases.")

tors have reported such growths from filtered extracts of leprosy nodules (both human and rat). It is believed by some that these various types (including a filtrable form) represent different phases in the life cycle of the leprosy bacillus which, in the human body, exists as an acid-fast tissue parasite, and multiplies only within the cells.

The relationship of these cultures to the true leprosy bacillus, however, is questionable. With some of these cultures transient granulomatous nodules have been produced in animals, and similar nodules have been obtained with other acid-fast bacteria also, such as the hay bacillus  $(M.\ phlei)$ . Furthermore, the inoculation of animals with human leprous material containing enormous numbers of organisms does not give rise to typical progressive lesions, and the question of the identity of any organism isolated must, therefore, remain open. For a critical review of the literature on this

subject see McKinley (Medicine 13, 377). In the opinion of McCoy, the true leprosy bacillus has never been cultivated with certainty, and the disease has never been transmitted artificially to animals or to man by direct inoculation of leprous tissue. Our knowledge of the behavior of the leprosy bacillus, therefore, is derived solely from observations of the disease.

Epidemiology.—The natural mode of infection is not known. Since the incubation period may extend over many years (usually from 2 to 10 or more) it is manifestly difficult to trace the transmission of the disease. Obviously it is only feebly contagious. There have been but very few cases among individuals working for many years in leper colonies, and even among people living in intimate contact with lepers in the same house the incidence is small, in spite of the fact that cases with open lesions are constantly discharging enormous numbers of organisms. All attempts to produce the disease in healthy individuals by inoculation with leprous material have failed with one questionable exception. The reasons for this apparently great resistance to the disease are obscure. Leprosy may occur in any race or in any climate, although according to Rogers a hot humid atmosphere seems to favor the spread of the disease. It is twice as common in males as in females, possibly owing to greater opportunities for contact. No age is exempt from the disease, but a majority of the cases occur between the ages of 10 and 40. Children are more susceptible than adults and some authorities believe that the infection is ordinarily acquired in infancy and remains latent until later life. Prolonged contact under unsanitary conditions appears to be an important factor. There is no proof that the disease is transmitted by insects. Mechanical transmission by insects might be possible, but no evidence of a cycle of development in insects has been found. The portal of entry is unknown. The frequent, although not invariable, occurrence of ulcerations at the junction of the bony and cartilaginous parts of the nasal septum in early cases has suggested that the infection may enter the body by this route, but this view is not generally held. It is possible also that the organisms may penetrate through an abrasion of the skin, as has been shown to occur in rat leprosy.

Pathology.—The specific lesion in leprosy is a granulomatous nodule, the leproma, which is formed in response to the presence of the leprosy bacilli. Unlike the tubercle, the lepromata never undergo caseation, possibly owing to the relatively feeble toxicity of the organisms. In these nodules the bacilli are usually present in enormous numbers, a few apparently lying free in the tissue spaces, but the great majority engulfed in large phagocytic "lepra cells." These cells are often grouped around the blood vessels, and may be literally stuffed with the bacilli. These are phagocyted in smaller numbers by the endothelial cells also. In the older nodules granular bodies termed globi are seen. These are composed of aggregations of bacilli and disintegrated cells packed in the lymphatic channels.

Clinical Picture.—Two types of leprosy are recognized clinically, nodular and nerve leprosy, although many cases eventually develop a combination of the lesions of each type (mixed leprosy).

In nodular leprosy the lepromata appear in the skin at times in successive crops, sometimes associated with the febrile paroxysms. At this time leprosy bacilli may be particularly numerous in the blood, and it has been suggested that the skin manifestations are the result of bacterial emboli in the capillaries. These granulomatous nodules develop in the corium and adjacent subcutaneous tissue, particularly about the arteries, sweat glands and hair bulbs. The epidermis is not involved primarily, but becomes thinned out and atrophied, and the mass may eventually ulcerate or disappear by

resolution. The mucous membranes of the nasopharynx may be studded with nodules which later ulcerate and discharge enormous numbers of bacilli. The regional lymph glands become enlarged but do not suppurate, and may contain organisms in almost as great profusion as do the skin nodules. The anterior part of the eye is frequently affected; occasionally the liver, spleen, testes and ovaries may show some infiltration. The lungs are rarely involved, and if acid-fast bacilli are found in the sputum, they must be differentiated from tubercle bacilli by guinea pig inoculation. The bacilli are sometimes present in the blood stream, free or within the leukocytes, particularly during the acute febrile exacerbations, and they may be sufficiently numerous to be found in blood films. They have been found in the urine and in sections of apparently normal tissue.

In nerve leprosy the lepromata surround and invade various peripheral nerves, most commonly the facial, ulnar and peroneal, producing areas of anaesthesia, paralyses, and trophic changes in the skin, bone, nails and underlying tissues. These changes are secondary to the degeneration of the axis-cylinders from the pressure of the specific lesion, and are not due to the presence of bacilli in the parts affected. Even in the granulomata the organisms are often relatively sparse and may be absent. They may be found in the nasal mucus, even when no ulceration is apparent. Nerve leprosy is a much less acute disease than nodular leprosy, and there is a greater tendency for the bacteria to die out in the nerve lesions.

In some cases the disease becomes arrested, and bacilli can no longer be demonstrated. These "burnt out" cases may be released from isolation but should be kept under observation, since relapses are not uncommon even after an interval of years.

Laboratory diagnosis.—In nodular leprosy the demonstration of M. leprae by stained smears is usually easy—in the skin nodules, the nose, the regional glands and even in the blood. In nerve leprosy, however, the organisms are very sparse, even in the granulomata about the affected nerves. In these cases, especially, examination of material from the nose may be the only means of establishing the diagnosis although it is often negative. The organisms may be found in the early exanthems that often precede the development of the characteristic features in both types of the disease, but not ordinarily in the anaesthetic macules secondary to nerve injury.

Smears may be prepared from the scrapings of any ulcerated nodule. In the absence of ulceration, material may be obtained by puncturing a nodule with a capillary pipette or needle, and squeezing until a drop of serum exudes. The organisms are usually present in abundance in smears from this fluid. If not found, the nodule may be incised with a small scalpel and scrapings made from the under surface of the skin. Recently the "skin clip" method has been used extensively. A small bit of skin about 2 mm. thick (not extending into the subcutaneous tissue) is excised with curved scissors, and smears are made from the under surface. Clips from the ear lobes have given a high percentage of positive results. Blisters can be produced on an affected area by CO<sub>2</sub> snow, and smears made from the accumulated fluid. Some prefer to excise a nodule and examine histological sections. These sections can be stained with haematoxylin to obtain a histological background before the acid-fast stain is applied. In staining the

leprosy bacilli the Ziehl-Neelsen method is used, decolorizing lightly with 20% aqueous sulphuric acid or 3% aqueous HCl.

The bacilli can be found in abundance in scrapings from ulcerated areas in the nose, and sometimes from the mucus when no lesions can be found. Some advocate scraping the intact mucous membrane. The organisms are more apt to be found when a coryza is present, and potassium iodide (60 grains) is often given to produce a drug coryza.

Material aspirated from enlarged regional glands usually contains an abundance of organisms.

Leprosy bacilli are present in the blood in sufficient numbers to be demonstrated directly in many cases of nodular leprosy, particularly during the febrile periods. They may be demonstrated in thick slide preparations as used in malaria. After dehaemoglobinization they are stained in the usual way. If none are found 5 or 10 cc. of blood may be collected in distilled water containing 1% of sodium citrate. After centrifuging, the sediment is treated with 10% antiformin at 37°C. for an hour. Centrifuge, wash the sediment to get rid of the antiformin, and smear on slides. In nerve leprosy examination of the blood is of little use, as organisms are rarely present.

In leprosy, as in other infectious diseases, the rate of sedimentation of the red blood cells is increased. Although the test has little diagnostic value, it is a useful index of the progress of the disease.

Rubino has shown that the serum of lepers causes agglutination and sedimentation of formolized sheep red blood cells within an hour. Confusing hetero-agglutinins in the sera are removed previously with non-formolized cells. The reaction appears to be specific, but it is not very sensitive. Since it is positive only in the well marked cases its usefulness as a diagnostic procedure is limited.

Allergic reactions.—Lepers develop hypersensitiveness to extracts prepared from the lepromata by heating a thin emulsion to 60°C. Injections of such preparations may give rise to reactions analogous to those produced in tuberculous cases by tuberculin. Intradermal injections into affected tissue may produce a local reaction within 48 hours, whereas in unaffected areas the reaction may be delayed. In advanced cases the reaction may not occur (Tisseuil). Moreover, similar reactions are obtained with tuberculin and similar preparations of other acid-fast bacteria, and even with sterile saline. At present, therefore, such reactions have little diagnostic value.

Skin lesions occur in lepers, particularly the so-called tuberculoid type, in which the bacilli are absent, and it is believed that they are allergic manifestations.

Wassermann reaction.—Many investigators have reported an unusually high incidence of positive reactions among lepers, particularly among those with the nodular type of the disease. The significance of this finding is disputed. Some authorities believe that this is accounted for by concomitant infections with syphilis or yaws, while others believe that in leprosy there are serological changes analogous to those occurring in syphilis and yaws. In many cases no evidence of either disease has been found, even at autopsy. McCoy has pointed out that the percentage of positive reactions among the lepers in the United States is also high, and that in these cases yaws can be excluded. The strength of the reaction fluctuates during the course of the disease, and is most often positive in the febrile exacerbations. Antisyphilitic therapy has not been of value in the absence of syphilitic lesions, and some workers have felt that it was actually harmful. In non-syphilitic lepers no changes have been found in the cerebrospinal fluid, and the Wassermann is negative. Kolmer has claimed that non-syphilitic lepers give a negative reaction with his modification of the complement fixation test, but others using this

#### DIPHTHERIA

technique have obtained approximately the same number of positive reactions as by the usual method.

M. leprae murium is the cause of a natural disease in rats (rat leprosy) which is prevalent in Europe, Asia, and America. Morphologically it is indistinguishable from M. leprae, and the lesions produced bear a close resemblance to those of the nodular type of human leprosy. Histological sections of the skin nodules show the same granulomatous infiltration in the corium and large rat lepra cells (histiocytes according to Oliver) packed with bacilli.

The disease can be transmitted to rats of the same species, and infection takes place as readily through skin abrasions as by subcutaneous inoculation. It is believed that the natural infection is acquired through the skin, possibly from bites. Mechanical transmission by rat fleas is possible, but no cycle of development has been demonstrated in any insect. The disease is not hereditary.

Attempts at cultivation of the organism have shown results similar to those obtained with M. leprae. Zinsser and Carey observed intracellular multiplication of the M. leprae murium in tissue cultures of growing rat spleen. Aside from this observation, however, the results reported are open to the same objections as those obtained in human leprosy.

In spite of the similarity of human to rat leprosy, most authorities believe that the diseases are distinct and that the organisms belong to separate species. Muir, Henderson and Landeman suggest that their relationship is analogous to that of the human and avian tubercle bacillus.

# NON-ACID-FAST BRANCHING BACILLE

#### 1) тритиврта

Corynebacterium diphtheriae (Bacillus diphtheriae). Discovered by Klebs, 1883, and cultivated by Löffler, 1884.—Diphtheria bacilli are present in large numbers in the characteristic ulcerations of the throat in diphtheria and in the secretions of the nose and pharynx. Infections of the nose, middle ear and larynx occasionally occur. The mucous membrane of the vagina or the conjunctiva may be infected. Skin wounds may become infected. A form of tropical ulcer designated Veld sore, common in various desert regions, has been shown to be due, in some instances, to the diphtheria bacillus. A diphtheritic angina may be complicated by a superadded infection with other organisms, particularly haemolytic streptococci or Vincent's organisms. In such cases the clinical picture may be misleading, and recognition of the underlying condition depends upon careful bacteriological studies.

Morphology.—The diphtheria bacillus is a slender, straight or slightly curved rod with characteristic beading or banding, and small, deeply staining polar bodies or metachromatic granules at either end. The latter may be seen in an 18 hour culture,

but are more abundant after 36 hours. They are brought out best with Neisser's stain with which the body of the bacillus is colored a pale buff, while the polar bodies stand out as dark blue dots. In culture the bacilli may show pear-shaped or clubbed forms. They are frequently arranged in a V or Y figure, and, what is most characteristic, show a palisade arrangement. Short chains may occur. It is Gram-positive, but not so tenacious of the gentian violet as are the cocci, and decolorization should not be carried too far. It is non-motile, and does not form spores.

Cultural characteristics.—The diphtheria bacillus grows best at 37°C. on media enriched with serum or blood. Löffler's blood serum is the medium most commonly used. (An egg medium made of the whole egg with glucose is equally good. Coagulated egg white or even a hard boiled egg answers fairly well.) The diphtheria bacillus grows so rapidly on this medium that it outstrips many of the contaminating bacteria. The colonies are at first small, whitish and opaque; later the center becomes heaped up

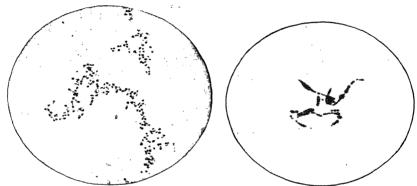


Fig. 13.—C diphtheriae stained by Neisser's method. (MacNeal.)

Fig. 14.—Diphtheria bacilli involution forms. (Kolle and Wassermann.)

and the edges characteristically crenated. This organism grows luxuriantly on blood agar and, unlike the diphtheroids, produces a narrow zone of haemolysis around the colonies. For isolation media containing potassium tellurite are recommended. This salt is reduced by the diphtheria bacillus, and the colonies, which are dark grey or black in the center with a lighter peripheral zone, stand out conspicuously. In broth it tends to grow on the surface and form a pellicle. It ferments the ordinary carbohydrates except lactose, saccharose and mannite, forming acid but not gas. Milk is not acidified or coagulated.

Variation.—Smooth, rough and intermediate types have been noted in cultures and also in the throats of convalescents. The S types lose their virulence in the transformation to the R forms. Typical, virulent S forms have not been produced experimentally from the R types. Smith and Jordan have obtained evidence of a filtrable form.

Toxin and toxoid.—The diphtheria bacillus in its growth in the body and in suitable culture media produces a soluble toxin which is responsible for the systemic manifestations and sequelae of the disease. The toxins

## DIPHTHERIA TOXIN

elaborated by different strains are antigenically similar, but the quantity produced varies greatly with the strain used, and with cultural conditions.

To prepare it, broth cultures of a suitable strain, known to be a good toxin producer, are made and incubated for about a week at 34°C. The medium is distributed in shallow layers in order to provide an abundance of oxygen which is needed for good toxin formation. The culture is then filtered and stored in a dark, cool place to retard deterioration. After a preliminary diminution in toxicity known as ripening, the toxin remains relatively stable under suitable conditions. A single strain, isolated by Park and Williams (No. 8) in 1895, is still used extensively for commercial toxin production. The strength of the toxin is expressed in terms of toxin units, one unit being the minimum quantity which will kill a 250 Gm. guinea pig in 4 days—the M.L.D. (minimum lethal dose).

In the deterioration of toxin, substances are formed called toxoids, which, although not in themselves toxic, are capable of neutralizing antitoxin, and which are antigenic. This change can be accelerated by the addition of formalin (0.2% to 0.4%). After several weeks incubation at 37°C. all of the toxin is converted into toxoid. The conversion is regarded as complete when 5 cc. injected subcutaneously or intraperitoneally into a guinea pig produces no effects. This toxoid is of great value in the active immunization to diphtheria.

Pathogenicity.—Diphtheria bacillus infections, whether in the throat or elsewhere, are almost invariably localized, and the systemic manifestations are due to absorption of the soluble toxin into the blood stream. Guinea pigs are very susceptible to the toxin and may be killed by as little as 0.00025 cc. Characteristic lesions are produced by the injection of the bacilli, which multiply locally and elaborate a toxin, or by the injection of the toxin itself. At the site of the inoculation there is an oedematous, inflammatory swelling, with enlargement of the neighboring lymph glands. The lungs are congested, and there are often haemorrhagic pleural and peritoneal effusions. The most characteristic finding is the enlarged and haemorrhagic adrenals.

Laboratory Diagnosis.—This depends upon identification of the organism in stained films from the exudate and from cultures made on Löffler's blood serum. It is often necessary to demonstrate the pathogenicity of the strain by means of virulence tests in guinea pigs.

Stained films.—On account of the distinctive morphology of the diphtheria bacilli it is possible to make an immediate provisional diagnosis in about 30% of the acute cases by demonstrating the organisms in smears from the local lesions. All antiseptic solutions should be discontinued several hours prior to obtaining the material for study. The smears should be stained with Löffler's methylene blue or by Neisser's method. It is often helpful to make a Gram stain also, taking care not to carry the decolorization too far, since diphtheria and pseudodiphtheria bacilli, and the fusiform bacilli of Vincent are the only Gram-positive bacilli likely to be found in the throat. The latter stain

readily with Löffler's stain, and are conspicuous on account of their size and distinctive morphology. The spirochaetes, with which they are always associated, are usually demonstrable with a good Löffler stain although they may require a stronger one. Recognition of these organisms is very important since fuso-spirochaetal infections may be indistinguishable clinically from diphtheria, or may complicate it.

Cultures on Löffler's medium should be made in all cases. Growth on this medium is rapid, and smears from the growth will usually show the diphtheria bacilli within 12 to 18 hours. If negative the cultures should be re-examined after 24 and again after 48 hours. Cultures from the nose and throat are also used to determine the period of isolation and to detect carriers. Since avirulent organisms are frequently present in normal throats it is often necessary to test the virulence of the organism found. For this purpose a pure culture must be obtained by plating, preferably on a tellurite blood agar plate.

Virulence tests.—Two cc. of a well grown 48 hour broth culture are injected subcutaneously into a young guinea pig weighing from 200 to 250 Gm. If the organism is virulent, death will occur in from 2 to 4 days with the characteristic pathological changes previously described. A control test should be made on a guinea pig which has previously received 500 units of diphtheria antitoxin.

If several strains are to be tested, one may give 0.2 cc. of the culture of each intradermally, following this 4 hours later with 100 units of antitoxin and observing the local lesion. A control pig which has been injected 24 hours previously with 500 units of antitoxin is inoculated in the same way. If the culture is virulent the test pig will develop an inflammatory reaction and later an area of necrosis about the puncture, while the control pig shows no reaction. Avirulent organisms produce no reaction in either animal. Several (up to 10) cultures may be tested simultaneously on the same pig in this way.

Antitoxin Unit.—The toxin produced by the diphtheria bacillus, when injected into animals, stimulates the production of a neutralizing antitoxin. The potency of an antitoxic serum is expressed in terms of antitoxin units. The unit, as originally defined by Behring, was the quantity which would protect a 250 Gm. guinea pig from 100 M.L.D. of a standard toxin. Since the toxin is unstable, it is less suitable for a permanent standard than is the antitoxin which can be preserved for long periods without deterioration. Antitoxin is now universally used as the permanent standard. In the U.S. the permanent standard is an antitoxin prepared by the National Institute of Health at Washington. The unit of this serum has been adopted arbitrarily and bears no relation to the original antitoxin unit of Behring and Ehrlich or to any "standard" toxin. Samples can be obtained by manufacturers by means of which they can determine the strength of their own (ripened) toxin preparations, and from this the potency of new antitoxic sera.

Standardization of toxin and antitoxin.—The strength of the toxin is measured by mixing various amounts with one unit of standard antitoxin, and determining the minimum amount of toxin which, in such a mixture, will kill a 250 Gm. guinea pig in exactly 4 days. This quantity of toxin is termed the  $L_+$  dose. The protocol as shown on page 87 illustrates such a test. The  $L_+$  dose of this toxin is 0.230 cc.

To standardize a new antitoxin, this L<sub>+</sub> dose of toxin is then mixed with varying quantities of the new antitoxin, until a mixture is obtained which will kill a 250 Gm. guinea pig in 4 days. This amount of antitoxin contains one unit, and the number of units per cc. can be calculated. The following protocol illustrates the titration of a

Dose of toxin, cubic centimeters	Dose of antitoxin	Death	Lesions
0.225	1 unit	5 da. 6 hrs.	Characteristic
0.230	1 unit	4 da. 4 hrs.	Characteristic
0.235	1 unit	3 da. 9 hrs.	Characteristic

new antitoxin with the  $L_+$  dose (0.23 cc.) of this toxin. In this test 0.0125 cc. of the new antitoxin protects for 3 days and 22 hours, and, therefore, contains one unit, or, by calculation, 80 units per cc.

Dose of toxin, cubic centimeters	Dose of antitoxin Death		Lesions	
0.23	(Control standard)  1 unit (Serum unknown)	4 da. 4 hrs.	Characteristic	
0.23	0.01 cc.	2 da. 10 hrs.	Characteristic	
0.23	0.0125 cc. 0.015 cc.	3 da. 22 hrs. 7 da. 6 hrs.	Characteristic Characteristic	

The  $L_+$  dose has no constant relation to the M.L.D. Precise determinations of the latter are more difficult, and are unnecessary for standardizing antitoxin. The amount of toxin which is exactly neutralized by one unit of antitoxin is termed the  $L_0$  dose. Theoretically, the difference between the  $L_0$  dose and the  $L_+$  dose should equal 1 M.L.D. Actually, however, this difference is considerably greater because toxin preparations contain other substances in large, but varying amounts, which neutralize antitoxin, but do not produce toxic effects in animals. In a neutral mixture, half of the antitoxin may be bound by these substances.

A reasonably accurate and economical method of standardization is to inject the toxin-antitoxin mixtures intracutaneously into guinea pigs and note the presence or absence of a local inflammatory reaction (Schick reaction).

An approximate preliminary standardization can be made by Ramon's flocculation test which depends upon the fact that in a series of toxin-antitoxin mixtures, flocculation occurs first in that mixture which is exactly neutralized.

Schick Test.—Most normal adults are immune to diphtheria owing to the presence of natural antitoxin in their blood. If a small amount of the toxin  $(\frac{1}{50} \text{ M.L.D.})$  is injected intracutaneously into immune individuals, it will be neutralized by the natural antitoxin, and no reaction will occur. If no natural antitoxin is present, there will result a local inflammatory reaction. This is known as the Schick test, and is of great value in the

determination of susceptibility and the effectiveness of immunization. Clinical observations have shown that the quantity of circulating antitoxin required to give a negative reaction protects an individual from the disease, even when virulent organisms are harbored in the throat.

The Schick test is performed as follows. Inject intradermally into the forearm 0.1 cc. of diphtheria toxin representing ½50 M.L.D. A positive reaction shows within 24 hours, and reaches its maximum intensity in 2 or 3 days as a reddened area 1 to 3 cm. in diameter with more or less induration. The intensity of the reaction is inversely proportional to the amount of natural antitoxin present. The reaction persists for about a week leaving a brownish pigmentation. It is important to inject an equal amount of the diluted toxin heated to 75° C. for one hour into the other arm as a control. A reaction occurring with the heated toxin is known as a pseudoreaction, and is regarded as an allergic response to certain constituents in the broth culture. It has nothing to do with susceptibility to diphtheria toxin since this is destroyed by the heating. It appears sooner than the true reaction, is more urticarial in type, and disappears more rapidly, usually within 48 hours. Combined reactions may occur and require considerable experience for their correct interpretation.

The great value of the Schick test is in the determination of susceptibility. The following table shows the percentage of susceptibles in different age groups. The low percentage of positive reactions under 6 months is due to a transient immunity inherited from the mother.

Average Susceptibility of Various Ages to Diphtheria (as Indicated by the Positive Schick Diphtheria-toxin Skin Test) in New York City (After Park)

Age	Schick + (Susceptible) Per Cent
Under 3 months	. 15
3 to 6 months	. 30
6 months to 1 year	. 60
1 to 2 years	. 60
2 to 3 years	. 60
3 to 5 years	. 40
5 to 10 years	. 35
10 to 20 years	
20 to 40 years	. 18
Over 40 years	. 12

The Schick test is also useful in suspected cases of diphtheria. The presence of virulent diphtheria bacilli in an ulcerative inflammation of the throat is not necessarily proof that the condition is diphtheria. If the Schick test is negative the patient may be a carrier, and the ulceration is probably due to some other type of infection. Although it may be possible for the diphtheria bacillus to cause local lesions in a person with natural antitoxin, toxic symptoms are absent, and serum therapy is not indicated.

Serum therapy.—In the preparation of antitoxin horses are injected subcutaneously with the toxin or broth filtrate at weekly intervals for three or four months. When each cc. of the serum is found to contain about 250 to 500 antitoxin units the horse is

bled from the jugular vein. Some sera contain as much as 1300 units in a cc. Methods of purifying and concentrating antitoxin are now generally employed which depend upon the principle that the antitoxin in the horse serum is precipitated with the globulins which come down on half saturation with ammonium sulphate. By thus eliminating other proteins, the total amount of foreign protein per unit is reduced and the dangers of a serum reaction correspondingly lessened.

Antitoxin should be given promptly as soon as the diagnosis is made. If the case is clinically suspicious it should be administered without waiting for the result of a culture. The statistics of Zingher indicate that the mortality approximately doubles with each day that the administration is delayed. In mild cases from 2500 to 5000 units, and in the more severe cases 10,000 units are given. In the malignant cases as much as 40,000 units should be used. It is usually given by intramuscular injection (not subcutaneously), but in severe cases 5000 to 10,000 units should also be given intravenously, particularly if the larynx is affected, or if administration has been delayed. It is desirable that the entire amount needed be given on the first day. Much larger dose are sometimes given, but it is probable, as Park believes, that nothing is gained by exceeding these quantities, or by repeating these doses daily. Tests for hypersensitiveness (see p. 253) should precede administration.

Immunization. Passive immunization with 500 units of diphtheria antitoxin is given to Schick-positive contacts when it is necessary to produce immunity quickly. This, however, protects only for 2 or 3 weeks.

Active immunization.—A strong, lasting active immunity can be produced in susceptible (Schick-positive) individuals by the injection of toxin-antitoxin mixtures or toxoid preparations. The toxin-antitoxin mixture is slightly under-neutralized and should contain about 85 to 90% of an  $L_+$  dose of toxin for each unit of antitoxin. The mixture should be prepared with a diphtheria toxin of such strength that each dose of 1 cc. of the finished mixture will contain approximately 0.1  $L_+$  dose of toxin almost neutralized with antitoxin. Three subcutaneous injections of 1 cc. each at intervals of about a week are required for immunization. This method is used less now than formerly on account of the possibility of sensitizing individuals to subsequent injections of horse serum.

When diphtheria toxin is treated with certain substances such as formalin, its toxic properties are destroyed by conversion into toxoid, while its immunizing ability is retained. This product has been used extensively in active immunization in the same way as the toxin-antitoxin mixture. It may, however, cause marked general reactions in older children and adults, owing to a hypersensitiveness to the proteins in the broth culture. These individuals give a pseudoreaction to the heated toxin used as a control in the Schick test. When this occurs the initial dose of the formol toxoid should be small, increasing the quantity if the reactions are not severe. If this reaction is very marked toxoid-antitoxin floccules (purified precipitate formed by the action of antitoxin on toxoid) can be used.

The addition of alum (2%) to toxoid increases its efficacy. Recent work by Park and others shows that a single dose of alum-precipitated toxoid confers immunity in over 90% of the cases.

The immunity produced by these methods develops within a few months and may persist for years. Maintenance of the immunity, however, appears to depend upon casual contacts with diphtheria bacillus carriers. The development of immunity is determined by the Schick test. For the complete control of the disease all children of

pre-school age with a positive Schick test should be immunized. The effective use of the antitoxin in treatment and particularly the immunization of susceptible children have reduced the mortality from diphtheria in New York per 100,000 from 86 in 1895 to 1899 to 2.6 in 1931.

Carriers.—Diphtheria is spread by convalescent and healthy carriers, and their detection is, therefore, of great importance. The organisms may occur either in the throat or nose, and both must be examined. Ordinarily a convalescent becomes free from the bacilli within a few weeks, but occasionally a chronic carrier state develops. The bacilli may lodge in the crypts of the tonsils, and tonsillectomy may be necessary to clear up the condition. It must be remembered, however, that some morphologically typical bacilli are not virulent, and that quarantine of cases showing only avirulent organisms is not justified. The pseudodiphtheria bacillus is a common inhabitant of the throat, but it can usually be distinguished morphologically from the true diphtheria bacillus. If any doubt arises the virulence must be tested.

Since 1927 outbreaks of a severe type of diphtheria which did not respond to antitoxin have been reported in Europe. Anderson (1931) isolated from one of these outbreaks an organism which he called *C. diphtheriae gravis*, which differed somewhat morphologically and culturally from the ordinary diphtheria bacillus. In some of these malignant epidemics an associated streptococcus infection of the throat was present. Some investigators have reported finding diphtheria bacilli in the internal organs in large numbers. Other hypotheses to explain these refractory cases have been suggested. There is no proof, however, that they are due to strains producing a toxin antigenically distinct from the ordinary toxin.

Corynebacterium pseudodiphthericum (B. hoffmanni).—This bacillus is a harmless saprophyte which is frequently found in healthy throats. It has no medical importance except that it must be distinguished from the diphtheria bacillus. This is usually possible by their morphological and cultural characteristics alone and always by guinea pig inoculation.

Pseudodiphtheria bacilli differ from true diphtheria bacilli in the following respects.

(1) They very rarely show the polar bodies in both ends, although they may show a dot at one end. (2) They tend to stain solidly, or at most with only a single unstained segment. They are shorter, thicker, stockier, and do not curve so gracefully. (3) They grow more luxuriantly, and are often chromogenic. There is no haemolysis on blood agar. (4) They produce very little acid on sugar media, not half that produced by true diphtheria bacilli. Some produce none. (5) They do not produce a toxin, and are not pathogenic for guinea pigs.

Corynebacterium xerose (B. xerosis).—This organism was first isolated from the conjunctiva of a form of chronic conjunctivitis known as xerosis, but since it is frequently present in the normal conjunctival secretion its pathogenicity is dubious. It resembles the diphtheria bacillus closely but differs in the following respects. (1) It does not usually show polar bodies with Neisser's stain. (2) It grows more slowly and delicately.

(3) It forms acid from saccharose and dextrose but not from dextrin. (4) It is not pathogenic for guinea pigs.

The following table gives the differentiation of these organisms by their fermentation reactions.

Species	Dextrose	Saccharose	Dextrin
C. diphtheriae C. xerose C. pseudodiphthericum	+	+	+

(Note.—One per cent sugar in Hiss serum-water media.)

Other diphtheroid bacilli have been isolated from many different sources—skin, urine, ascitic fluid, spleen, lymph nodes, etc. Their presence in the lymph nodes has led to the belief that they were the etiological agent in various diseases—Hodgkin's disease, arthritis, leprosy, etc. They are found so frequently in normal tissues, however, that the assumption that they are the cause of any specific disease from this finding alone is unwarranted.

#### GLANDERS

Actinobacillus mallei (Bacillus mallei) (Glanders bacillus) Löffler and Schutz, 1882.—This is the cause of a rather common disease of horses. When affecting the superficial lymphatic glands, it is termed "farcy"; when producing ulceration of the nasal mucous membrane, it is called "glanders." This is often followed by involvement of various organs, especially the lungs.

In man there are 2 types of glanders—chronic and acute. In the chronic form an abrasion becomes infected from contact with glanders material and an intractable foul discharging ulceration results. There may be an involvement of the nasal mucosa as well as skin. This may persist for months with lymphatic involvement or may become acute. The acute form may also develop from the start, and the cases are usually regarded as pyaemia. There is great prostration with marked pains in the extremities. Pustular lesions, resembling those of smallpox, may be present. Acute glanders is almost invariably fatal.

Morphology.—The bacillus is a narrow, sometimes slightly curved rod, varying greatly in size, but averaging about  $3 \times 0.3\mu$ . It is non-motile and Gram-negative. It often presents a beaded appearance, and tends to stain irregularly. It stains readily, but is easily decolorized. The best stains are carbol thionin and carbol fuchsin. In sections stained with carbol thionin the bacilli are apt to be decolorized by the passage of the section through the alcohols. This decolorization may be avoided by blotting carefully after the thionin, then clearing with xylol or some oil, and mounting. Nicolle's tannin method is good.

Cultural characteristics.—The optimum temperature for growth is 37°C. In primary cultures from pus or tissues the colonies may not appear for 48 hours. The growth is

at first somewhat like that of the typhoid bacillus, later it becomes yellowish and more opaque. The addition of glycerin to the medium enhances the growth. Broth cultures show a slimy sediment and a surface pellicle. Milk is slowly acidified and coagulated. The characteristic growth is on potato. At first this is light brownish or yellowish, honey-like or mucilaginous. By the end of a week it has a cuprous oxide-like reddish tint with greenish borders, and the potato assumes a dirty brown color. Only the pyocyaneus bacillus and the cholera vibrio give a similar discoloration of potato, and these are easily differentiated.

Animal inoculation.—If the material is injected intraperitoneally into a male guinear pig, marked swelling of the testicles occurs within a few days (2 to 10), the Strauss reaction. Cultures should be made from the swollen testicle. The glanders bacillus can often be isolated from contaminated material in this way.

Laboratory diagnosis is made by isolating and identifying the glanders bacillus culturally or by guinea pig inoculation from the lesions. Blood cultures are usually negative. Cultures of glanders bacilli are the most dangerous of all laboratory cultures, and should be handled with extreme care.

Mallein is a preparation allied to tuberculin, and is used in a similar way for the diagnosis of glanders in animals. It is prepared by sterilizing cultures that have grown in glycerin broth for about 5 months by means of heat (100°C.). The supernatant fluid is filtered through a Berkefeld filter, evaporated to  $\frac{1}{2}$ 3 of its original volume, and subsequently made up to its original volume with a 10% glycerin solution containing 1% carbolic acid. One cc. of the prepared solution is injected subcutaneously into the animal. The reaction consists of a rise in temperature and local oedema. Conjunctival instillation of mallein is also used.

Agglutination and complement fixation tests are also used for diagnosis.

Actinobacillus pseudomallei (Bacterium whitmori).—This organism is the cause of a rare, glanders-like disease, described by Col. Whitmore of the British Army, found at autopsies of beggars in Rangoon. Stanton and Fletcher suggested the name melioidosis, in order to describe its close relationship to glanders. A. pseudomallei closely resembles A. mallei, being a small Gram-negative bacillus about the same size and shape, and occurring in very large numbers in the acute lesions of the disease.

In culture it closely resembles the glanders organism, but it is more actively motile and liquefies gelatin more rapidly. It grows luxuriantly and forms a dense wrinkled culture on glycerin agar. In guinea pigs the infection is more rapidly fatal than is glanders, producing in the male guinea pig an acute orchitis—the so-called Strauss reaction. The organism is excreted in the urine and faeces of infected laboratory animals. Several cases of natural infection have been found in rats, and one case in a domestic cat has been observed by Stanton.

A. mallei and A. pseudomallei are closely related serologically.

## CHAPTER V

# STUDY AND IDENTIFICATION OF BACTERIA—GRAM-NEGATIVE BACILLI. KEY AND NOTES

KEY to the recognition of non-spore-bearing, non-chromogenic, Gramnegative non-branching bacilli.

I. Do Not Grow on Ordinary Media.—Minute rod-shaped cells, sometimes thread forming and pleomorphic. Grow best (or only) in the presence of haemoglobin and in general require blood serum, ascitic fluid, or certain growth accessory substances. Non-motile.

## A. Aerobic.

- (a) Affecting the respiratory tract.
  - (1) Hemophilus influenzae (Pfeiffer influenza bacillus).
  - (2) Hemophilus hemolyticus. Haemolysis in blood broth. Found in upper respiratory tract.
  - (3) Hemophilus pertussis (Bordet-Gengou bacillus of whooping cough).
- (b) Affecting the conjunctiva.
  - (4) Hemophilus conjunctivitidis (Koch-Weeks bacillus of acute infectious conjunctivitis).
  - (5) Hemophilus lacunatus (Morax-Axenfeld bacillus of subacute infectious conjunctivitis or "angular conjunctivitis").
- (c) Affecting the genital region.
  - (6) Hemophilus ducreyi (Ducrey's bacillus of chancroid).
  - (7) Hemophilus canis (preputial secretions of dogs).

### B. Anaerobic.

(1) Dialister pneumosintes (Bact. pneumosintes of Olitsky and Gates. Occurs in the nasal secretions. Very short rods with pointed ends occurring singly and in pairs. Strict anaerobe.

# II. Grow Scantily or Require Enriching Substances in Media.

- (1) Pasteurella tularensis (Bact. tularense). Growth occurs only on enriched media containing egg yolk, cystine or blood agar.
- (2) Brucella melitensis (Bact. melitense, Bruce) causes Malta fever (brucellosis). Distributed usually through goat's milk.
- (3) Brucella abortus (Bacl. abortum, Bang) causes contagious abortion in cattle, and brucellosis in man.
- (4) Brucella suis (Traum) causes contagious abortion in swine, and brucellosis in man.

## III. Grow Well on Ordinary Media.

A. Cultures in litmus milk PINK.

(Commonly occur in intestinal canal of man and animals. Produce acetyl-methyl-carbinol—Voges-Proskauer reaction.)

- (a) Non-motile.
  - (1) Aerobacter aerogenes (B. lactis aerogenes) produces acid and gas in adonitol, lactose, dextrose and sucrose. No action on dulcitol. Indol not formed.
- (b) Motile.
  - (2) Aerobacter cloacae (B. cloacae) ferments lactose and sucrose. No action on adonitol or dulcitol. Indol formation indefinite. Slow liquefaction of gelatin. Commonly occurs in intestinal canal of man and animals. Generally attacks carbohydrates, forming acid and often gas composed of CO<sub>2</sub> and H<sub>2</sub>. Acetyl-methyl-carbinol formed from dextrose.
  - (3) Escherichia (B. coli group). Representative type species are:
    - (a) Escherichia coli (B. coli communis). Acid and gas are formed from glucose, lactose and dulcitol; not from sucrose. Indol produced. A "commensal" species.
    - (b) Escherichia communior (B. coli communior) similar to E. coli except that acid and gas are formed from sucrose.
- B. Cultures in litmus milk LILAC.
  - (a) Non-motile.
    - (1) No gas generated in dextrose or lactose broth.
      - (aa) Haemorrhagic septicaemia group.
        - (1) Pasteurella pestis (B. pestis). Causative organism of plague in man, rats and ground squirrels. Oval bacilli with tendency to bipolar staining. Agar colonies grayish white, translucent, iridescent, undulate. Several species of the genus Pasteurella are pathogenic for domestic and wild animals (fowl cholera, swine plague, etc.).
      - (bb) Dysentery group. Attack a number of carbohydrates with formation of acid, but no gas.
        - (1) Shigella dysenteriae (B. dysenteriae, Shiga). The cause of the bacillary dysentery in man.
        - (2) Shigella paradysenteriae (Hiss, Flexner, Strong types). The cause of dysentery in man, especially of the so-called "Summer diarrhoea," of infants.
    - (2) Gas generated in dextrose and sometimes lactose broth.
      - (aa) Friedländer group. Ferment a number of carbohydrates with formation of acid and gas. No liquefaction of gelatin. Encapsulated. Found principally in respiratory tract of man.
        - Klebsiella pneumoniae (Pneumobacillus, Friedländer). Agar colonies white, very viscid, opaque, shiny, convex. Associated with pneumonia and other inflammations of the respiratory tract.
        - (2) Klebsiella granulomatis (Calymmatobacterium granulomatis). Closely resembles Friedländer's organism. Found in lesions of granuloma venereum.
        - (3) Klebsiella rhinoscleromatis. Given as cause of rhinoscleroma.
        - (4) Klebsiella ozaenae (B. ozaenae). Given as cause of ozaena. Infectious for house and field mice.

- (b) Motile.
  - (1) Do not liquefy gelatin.
    - (aa) Typhoid group. Attack a number of carbohydrates with formation of acid but no gas. Do not reduce neutral red.
      - (1) Eberthella typhosa (B. typhosus, Eberth.). The cause of typhoid fever. Pathogenic for laboratory animals by subcutaneous or intravenous injection. Indol not produced. Agglutinated by immune serum.
    - (bb) Salmonella Group. Attack numerous carbohydrates with formation of acid and gas. Acetyl-methyl-carbinol not formed. Reduce neutral red. Occur in intestinal canal of man and animals. Certain types pathogenic. Cause food poisoning and paratyphoid fevers in man. Differentiation depends upon separate identification of antigenic constituents with special immune sera.
      - (1) Salmonella schottmülleri (B. paratyphosus B). No acid or gas in lactose broth. The cause of paratyphoid fever. Occurs in food poisoning where meat from infected animals is used. Darkens lead acetate.
      - (2) Salmonella aertrycke (B. aertrycke). Encountered in continued fevers of paratyphoid type. Meat poisoning.
      - (3) Salmonella suipestifer. Reported in epidemics of food poisoning and in typhoid-like fevers.
      - (4) Salmonella enteritidis (B. enteritidis, Gaertner). First isolated from intestines in epidemic of meat poisoning. Occurs in domestic and wild animals.
      - (5) Salmonella paratyphi (B. paratyphosus A.). Does not change lead acetate.
      - (6) Salmonella morgani (Bact. morgani). Found in intestinal canal of dysentery patients.
  - (2) Liquefy gelatin.
    - (aa) Proteus group. Highly pleomorphic rods, filamentous and curved. Produce characteristic amoeboid colonies on moist media and decompose proteins. Ferment dextrose and sucrose but not lactose. Do not produce acetyl-methyl-carbinol.
      - (1) Proteus vulgaris—Liquesies gelatin rapidly.
      - (2) Proteus mirabilis-Liquesies gelatin.
      - (3) Proteus asiaticus does not liquefy gelatin.

Note.—Proteus  $X_2$  and  $X_{19}$  used in Weil-Felix reaction are strains of P. vulgaris isolated from urine of patients suffering from typhus fever.

# GRAM-NEGATIVE BACILLI REQUIRING SPECIAL MEDIA

Hemophilus influenzae (Bacillus influenzae) Pfeiffer, 1892.—This is the type species of the so-called haemophilic bacteria, most of which grow only on media containing special food factors present in haemoglobin.

Morphology.—H. influenzae is a small bacillus (0.5 by 0.25µ), non-motile, and Gramnegative. In cultures it frequently shows involution forms, cocco-bacillary or filamentous forms. The latter have been noted particularly in strains isolated from the

meninges. These organisms stain rather faintly, sometimes more deeply at either end so that they resemble diplococci. They are best demonstrated by staining for 5 minutes with a 1-10 dilution of carbol-fuchsin, or with Löffler's methylene blue.

Culture.—The influenza bacillus can be isolated on plates of rabbit's blood agar (human blood may be slightly inhibitory), or on Avery's sodium oleate haemoglobin agar which inhibits contaminating organisms and favors the growth of the influenza bacillus. Chocolate agar or chocolate broth are particularly favorable. The colonies are very minute, transparent and dew-drop like, and are barely visible without a lens during the first 24 hours. They are invisible by transmitted light.

Two growth-promoting constituents must be present in the media to obtain a growth of the influenza bacillus. One factor, designated X, is an iron compound derived from haematin. This is thermostable and acts like a peroxidase. The other factor, V, is present in blood and also in certain vegetables, especially potato, and in yeast. It is thermo-labile. It has been suggested, but not proved, that this factor is a vitamin. It is produced by certain bacteria, and it is for this reason that the influenza bacillus grows more luxuriantly in the neighborhood of colonies of other organisms, (especially the staphylococcus, G. tetragena etc.),—the so-called "satellite phenomenon." The H. influenzae is more exacting in its requirements for these accessory food factors than are most of the other members of this genus.

Many of the pathogenic strains produce indol, and some strains reduce nitrates to nitrites. Occasional strains, especially those isolated from cases of meningitis in children, are haemolytic. These tend to be very pleomorphic and often show long filaments or threads. Some of them do not require the X factor for growth. Smooth (S) and rough (R) forms occur, and the transformation from one type to the other can be effected by animal passage or by cultivation. The R types are less virulent and show more pleomorphism.

Virulence.—Influenza bacilli have, with some exceptions, very little virulence for animals. Parker has shown that culture filtrates of some strains produce a soluble toxin which, if injected in large doses, will kill rabbits within one and one-half hours.

Fothergill et al (1937), however, were able to increase greatly the virulence for mice by suspending the organisms in mucin solution and inoculating intraperitoneally. With smooth strains recently isolated from cases of meningitis they caused progressive, fatal infections with bacteriaemia, and could enhance the virulence by serial passages.

Influenza bacilli appear to be antigenically heterogeneous. Except for certain strains from cases of meningitis reported by Rivers and Kohn, no definite grouping by means of serological reactions has been demonstrated.

Occurrence.—Influenza bacilli are frequently found in the healthy throat and nose. They may cause sinusitis, otitis media, angina, bronchitis and bronchopneumonia, either alone or associated with some other organism. They are frequently present in tuberculous lesions of the lungs and in bronchiectatic cavities. They may cause a subacute bacterial endocarditis indistinguishable from that due to the S. viridans. They have been found as terminal invaders in the blood of patients dying from unrelated conditions. A fatal type of meningitis in children is caused by influenza bacilli. Strains isolated from this condition are often atypical in morphology, biochemical and other reactions, and are serologically related (Rivers and Kohn).

Relationship to influenza.—H. influenzae was originally isolated by Pfeiffer from the sputum and nasal passages of cases of influenza, and was

believed by him to be the etiological agent of the disease. Recent work, however, has established the fact that a filtrable virus is the primary cause of the disease. Shope has shown that typical swine influenza can be produced in swine only when the influenza bacillus is inoculated together with the virus of influenza (human or swine) and it is possible that a similar relationship may exist in man. On the other hand, the disease can be transmitted to ferrets by inoculation with the swine or human virus alone, and is not modified by the addition of either the human or porcine influenza bacillus.

Lewis and Shope have applied the term *Hemophilus influenzae-suis* to strains isolated from swine influenza.

Hemophilus conjunctivitidis (Koch-Weeks bacillus) Koch, 1883.—This organism produces a severe acute conjunctivitis which is highly contagious. It is particularly

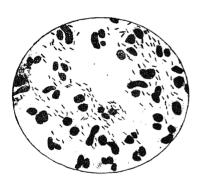


Fig. 15.—The Koch-Weeks Bacillus. (Hansell and Sweet.)



FIG. 16.—The diplobacillus of Morax in the exudate of conjunctivitis. (From McFarland after Rymowitsch and Matschinsky.)

common in Egypt and the Philippines. The conjunctivae of animals, other than man, resist infection with this bacillus. Flies are an important factor in its transmission in Egypt. The incubation period of the disease is short, 12 to 36 hours.

Smears from the conjunctival secretion show large numbers of small Gram-negative bacilli, chiefly intracellular. They resemble *H. influenzae* morphologically and culturally, and are regarded by many as identical.

Hemophilus lacunatus (Diplobacillus of Morax).—This organism causes a mild chronic conjunctivitis, chiefly at the inner angle of the eye, and may produce a keratitis.

The bacilli are about 1 or  $2\mu$  by about  $1\mu$  in width, and tend to occur in pairs or short chains. They are non-motile and Gram-negative. They grow only on media (preferably slightly alkaline) which is enriched with blood or serum. On Löffler's medium they form, within 24 hours, little pits of liquefaction which tend to become confluent

later. This is regarded as fairly characteristic. Neither the X nor V factor is necessary for its growth.

Note.—A Gram-negative bacillus which is less than r micron long, growing singly, or in pairs, and known as the bacillus of Zur Nedden has been stated to produce corneal ulcers. It grows readily on agar and other ordinary culture media. It coagulates milk.

#### CHANCROID

Hemophilus ducreyii (Bacillus of chancroid) Ducrey, 1889.—This is often called the streptobacillus of Ducrey because it occurs in chains, but when ulceration has set in, chain-formation is not observed.

The organism is about 0.5 by  $1.5\mu$ , is Gram-negative, non-motile, and forms no spores. It stains irregularly, often more intensely at the poles. It is found in the granulation tissue in the base of the ulcers and in the pus, often within leukocytes. Aspirating the gland juice with a hypodermic syringe from the buboes which are apt to follow chancroid offers the best means of obtaining pure cultures. This should be done before the bubo suppurates. Since Ducrey's bacillus is exceedingly delicate. the syringe should be warmed to body temperature and the transfer made to media at the same temperature and at once put in the incubator. Cultures kept at room temperature quickly die, but remain alive in the incubator for a week or more. Cultures and smears can also be made from the lesion, preferably before it ulcerates. Teague and Diebert got 140 positive cultures in 274 cases by the following method: Blood from a rabbit's heart was deposited in small tubes in 1 cc. quantities. After the blood had clotted, the tube was heated at 55°C. for 5 minutes to destroy natural bactericidal substances. The serum about the clot was inoculated with scrapings from the edge or floor of the ulcers or pus from a bubo and after 24 hours' incubation smears were made and stained for small, Gram-negative bacilli in chains. Small glistening grav colonies. which slide over the surface when touched with the platinum loop, may be grown by transfer to blood agar plates.

Intraculaneous diagnostic tests have been employed, using either pus from a bubo or culture suspensions. Sanderson and Greenblatt (1937) report clinically correct results in 96% of a series of about 75 cases and 100 negative controls. They introduced 1 cc. of sterile defibrinated human blood into a tube of solidified beef-infusion agar and inoculated this with 0.1 cc. of a growing culture. This was incubated 48 to 72 hours under reduced O tension obtained by warming the tube to expel part of the air and sealing with paraffin. The blood was then removed and the sediment washed twice with 25 cc. of sterile distilled water to remove haemoglobin. The sediment was suspended in salt solution, killed by heating 30 minutes at 60°C., and 1-10,000 merthiolate added. To make the test, 0.1 cc. was injected intracutaneously and the result observed after 48 hours.

## WHOOPING COUGH

Hemophilus pertussis (Bacillus of Bordet-Gengou).—This bacillus was reported as the cause of whooping cough by Bordet and Gengou in 1906. This relationship has been questioned repeatedly, and the disease has been regarded by some as due to a filtrable virus, but at present the evidence

seems to be in favor of the former view. It can be demonstrated by stains and by culture in the thick sputum in the early stages of the disease.

Morphology.—The bacillus is small  $(.3 \times 1.2\mu)$ , ovoid and shows bipolar staining. It resembles somewhat the influenza bacillus. It is Gram-negative and non-motile. Capsules can be demonstrated in freshly isolated strains.

Cultures.—For primary cultures blood or ascites agar or, preferably, a special potato glycerin blood agar medium is used. Cultures can be made from the sputum in the ordinary way, or by holding a plate of the medium in front of the mouth of the patient during a paroxysm of coughing. The colonies develop rather slowly, at first resembling those of influenza bacilli, and after 48 hours becoming slightly larger and thicker. There is a slight zone of haemolysis around the colonies. The bacillus does not ferment any of the carbohydrates. It remains viable, and grows slightly at low temperatures, unlike the influenza bacillus. After cultivation on artificial media for a time it can be made to grow on plain media, and, unlike the influenza bacillus, it does not require the V and X factors for growth.

Variation.—Leslie and Gardner (1931) studied the antigenic properties of 32 strains, and divided them into four groups which they designated as Phases 1, 2, 3, and 4. The freshly isolated (smooth) strains were of Phase 1, and were toxic for animals, while the old (rough) strains fell into Phases 3 and 4, and were non-toxic. Furthermore, they were able to produce all four phases from a single strain by cultural methods, showing that the types which have been previously described were merely variants. They have also shown that vaccines made from the S forms only were effective in immunizing. All recently isolated (smooth) strains appear to be identical in antigenic structure.

Vaccines made from the bacillus have been used extensively in prophylaxis and treatment. Reports of their value have been conflicting. It seems probable that this is due to the fact that some of the vaccines used may have been made from strains which had become dissociated.

Complement fixing and agglutination reactions have been obtained, but have not been of use in diagnosis.

Dialister pneumosintes (Bacterium pneumosintes).—During the pandemic of influenza in 1918 Olitsky and Gates obtained a small, Gram-negative, anaerobic bacillus by inoculating rabbits intratracheally with material from the nose and throat of early cases of influenza. This produced a febrile disease with leukopenia and small areas of hacmorrhage or oedema in the lungs. Specific agglutinins of low titer were noted in the blood of the infected animals. On account of its small size, especially in young cultures, this organism may pass through the coarser bacterial filters. It can be cultivated on media enriched with fresh animal tissue under strictly anaerobic conditions.

This organism has also been isolated from the throats of normal individuals, and there is no evidence of any etiological relationship to influenza or other pathological condition.

### BRUCELLOSIS

Brucella melitensis Bruce, 1887. (Caprine type.) Brucella abortus Bang, 1897. (Bovine type.) Brucella suis Traum, 1914. (Porcine type.) Brucellosis primarily affects goats, cows, and hogs, causing abortion; secondarily, these infections gain entrance to man, in whom the symptoms are practically the same whether the infection is of goat, cow or hog origin. The disease in man has been called undulant fever on account of the successive waves of pyrexia which may extend over several months.

Alice C. Evans, in 1918, discovered the close serological relationship of the organisms of Bruce and Bang, thus laying the foundation for world recognition of the relationship of the three infections. Meyer and Shaw proposed the generic name Brucella in 1920.

Morphology.—The three organisms cannot be differentiated morphologically, although freshly isolated melitensis cultures seem most like coccoid bacilli, averaging  $.5 \times .5$  to  $2\mu$ , while suis is the longest, and abortus is of intermediate length. All are Gram-negative, non-motile, and non-spore forming.

Cultural characteristics.—The organisms grow well on ordinary laboratory media, especially glucose infusion agar, liver agar, or serum agar adjusted to a pH of 6.8 to 7.4 and incubated at  $37^{\circ}$ C. In primary cultures the colonies become visible in 3 to 6 days and are small, dew-drop like, later becoming opaque and raised. In broth there is a diffuse turbidity. None of the carbohydrates are fermented. The organisms are killed by heating to  $60^{\circ}$ C.

Differential Characters.—Likeness of the three species has stimulated intensive efforts to discover differences which would serve to identify a species irrespective of the host from which it was isolated. Differences have been sought in carbon dioxide requirements, serological relationships, bacteriostatic action of dyes, hydrogen sulphide production, and glucose utilization.

Increased carbon dioxide tension .- Br. abortus was originally isolated by Bang who inoculated material from the uterus of an aborting cow into deep tubes of serum agar in which colonies developed only in a zone of partial oxygen tension 1 cm. below the surface. Huddleson found that atmospheric air containing approximately 10% CO2 was suitable for original isolation of Br. abortus. After freshly isolated cultures of Br. abortus have been subcultured about ten times, they become trained to grow freely thereafter in ordinary atmospheric air, and the CO2 requirement cannot be restored to them by long residence in an artificially infected animal. Furthermore a recently isolated Br. abortus culture cannot be made to lose its CO2 requirement of isolation by long residence in a goat, which is the normal host of the air-growing Br. melitensis. Since normal strains of Br. abortus cannot be isolated originally in atmospheric air but only under increased CO2 tension, the important differential character is established that a brucella organism which requires carbon dioxide for original isolation is Br. abortus. There are, however, strains of Br. abortus in cattle and man in Rhodesia which grow freely in normal air from the start, but the question arises as to whether they may not be vaccinal strains derived from living vaccine used for protecting cattle against contagious abortion. Br. melitensis and Br. suis do not require CO2 for original isolation or for subsequent growth, although both species are susceptible of isolation in 10% CO2 and of subcultivation under increased carbon dioxide tension.

Serological relationships.—The three species are agglutinated equally by an antiserum prepared from any one of the three; therefore the species cannot be differentiated by simple agglutination tests. Br. abortus cannot be differentiated from Br. suis by agglutinin absorption tests, but Br. abortus or Br. suis on the other hand can, in most instances, be differentiated from Br. melitensis by agglutinin absorption. However, there still remains a large number of Br. abortus cultures which cannot be distinguished by agglutinin absorption from Br. melitensis. The conclusion is that agglutinin absorption is a very unreliable procedure for differentiation of the three species. Tularemia sera agglutinate the Brucella oftener than brucellosis sera agglutinate P. tularensis, but in any case a correct differentiation between genera can be made by agglutinin absorption.

Bacteriostatic action of dyes.—Huddleson and Abell have been able to separate the three types by the inhibitory effect on growth which certain dyes exercise when incorporated into a culture medium of beef liver agar, as shown by the following table:

	Methyl violet 1:100,000	Basic fuchsin 1;25,000	Pyronin 1: 200,000	Thionin 1: 50,000
Br. suis.	No growth.	No growth.	No growth.	Growth.
Br. abortus.	Growth.	Growth.	Growth.	No growth.
Br. melitensis.	Growth.	Growth.	Growth.	Growth.

Hydrogen sulphide production.—Huddleson, Hasley and Torrey have shown that, depending upon the amounts of available sulphur in the culture medium, Br. snis produces hydrogen sulphide for the longest time, Br. abortus less, and Br. mclitensis not at all. The test is made by suspending a strip of dry lead acetate paper in the culture tube above the liver infusion agar at the time of inoculation and observing the degree of darkening during four days.

Glucose utilization.—McAlpine and Slanetz have shown by chemical analysis that Br. melitensis and Br. suis utilize the glucose in the culture medium while Br. abortus does not.

Cultures Isolated from Man in the United States.—Human cases of brucellosis have been reported from all of the states, about 1800 being reported annually in recent years by state departments of health, principally on the basis of positive agglutination tests.

Br. melitensis has been isolated almost exclusively from residents of Texas, New Mexico, Arizona, or California, where goat raising is a prominent industry.

Br. abortus, because of its CO<sub>2</sub> requirement for isolation, has doubtless been missed in those laboratories in which CO<sub>2</sub> apparatus is not a part of the routine equipment used for blood cultures. Gilbert and Coleman working in the New York state laboratory incubated duplicate samples of patient's blood and cow's milk in normal air and in an atmosphere containing 5 to 10% of carbon dioxide. Of 88 cultures isolated (44 from human blood and 44 from cow's milk) all showed primary growth in the CO<sub>2</sub> environment, while practically none gave primary growth in normal air, thus indicating that

only the bovine (abortus) type is prevalent in that state, where few hogs are raised. Huddleson isolated 57 cultures from man in Michigan, only 2 of which were *Br. suis*. The small number of these corresponds to the small extent of the hog raising industry in Michigan.

Br. suis was the type in 69 of 104 cultures isolated from man in Iowa, 35 being Br. abortus. All were isolated by Hardy, Borts and Jordan in the State laboratory where duplicate samples were incubated in air and in CO<sub>2</sub>. The high percentage of Br. suis cultures corresponds to the prominence of the hog-raising industry in Iowa. Beattie and Rice reported a milk-borne epidemic of 30 cases in Council Bluffs, Iowa, confined to city users of raw milk supplied by one dairy man. Br. suis was cultured directly in normal air from the blood of 6 of the 30 patients and obtained from cream of the milk of one cow in the dairy by guinea pig inoculation. This cow before purchase had associated with aborting hogs on another farm. Br. suis was the classification of 32 of 33 cultures isolated by Sellers of the Georgia State laboratory from human blood. which were all incubated in normal air, none in CO2 because of the lack of suitable apparatus. Whether the failure to isolate Br. abortus was due to the failure to use  $C(\cdot)$ . apparatus remains a question. The epidemiological evidence, however, points to drinking raw cow's milk containing Br. suis as the cause. Evidence of the spread of Br. abortus from cows to hogs by contact is lacking, but the spread of Br. suis from hogs to cows by contact is amply demonstrated.

Pathogenicity for laboratory animals. In monkeys a disease resembling human brucellosis can be produced with caprine and porcine strains but less regularly with the bovine type. Ordinary laboratory animals are also less susceptible to bovine strains. Inoculation into guinea pigs (preferably males) may produce a low grade infection of low mortality. After 2 or 3 months there is enlargement of the spleen, which is filled with small greyish, tubercle-like nodules. The liver and lymph glands may also be affected, and there is often a caseous epididymitis.

Brucellosis in man.—The disease is contracted by drinking raw milk from diseased cows, by contact with infected animals, or by handling infected meat. It may be spread by cheese and butter, since the souring of milk does not destroy the organism. Fifty-seven laboratory infections have been recorded in 17 laboratories in the United States. Infection may occur through the skin as well as through the alimentary tract. The spread of the disease through carriers has not been demonstrated. The results of agglutination tests on individuals with no history of the disease, especially veterinarians and butchers, suggests that an immunity has been acquired from subclinical or unrecognized infections. The incidence of Br. abortus infections is low in proportion to its frequency in cattle, and many of the cases are very mild. The caprine and porcine types are more pathogenic for man.

The mortality from the disease is low, 2 to 3%, but the pyrexia may last for weeks or months. In many cases of melitensis infection there are regularly recurring periods of fever lasting from one to three weeks, but often quite uniform in the same individual. Each cycle is characterized by a preliminary period of daily step-like rise in temperature and sharp morning remissions; a period of high continued fever; and a period of step-like descent. These may follow each other in close succession for months, or they may be separated by varying periods of normal temperature. A similar course is seen in some cases of abortus infection, but more often the temperature is irregularly elevated. Relapses are less frequent than in the melitensis cases. The pulse is relatively slow. The blood shows a leukopenia with a relative lymphocytosis. The spleen and sometimes

the lymph glands are enlarged. Muscle and joint pains are common. In long continued cases there is a tendency to localization of the infection in some particular area. The spleen, lymph glands, and intestinal tract may harbor the organisms after the general infection subsides. One or more joints may be involved, and the organisms have been cultivated from the synovial fluid. Suppurative lesions of the testicle, epididymis, ovary, gall bladder and appendix have occurred. Several cases of meningitis have been reported in which the organism was found in the cerebrospinal fluid. Neuritis (especially sciatica) is often noted. The early morning sweats may suggest phthisis. These clinical features are not very distinctive, and the diagnosis depends upon laboratory investigations.

Laboratory diagnosis.—Blood cultures should be taken at the onset of a febrile paroxysm. The organisms are present in the blood early in the disease in a large proportion of the melitensis cases, less often in the abortus cases. They may be present even after the fever has subsided. It is essential to incubate part of the culture in an atmosphere containing 10% carbon dioxide if Br. abortus is suspected.

Methods for making these cultures are described in the Chapter on blood cultures. Growth usually appears in from 5 to 10 days, but the cultures should not be considered negative for at least 4 weeks, since the primary growth of these organisms may be slow. Porcine and caprine strains are more easily isolated than bovine types and will grow both aerobically and under increased CO<sub>2</sub> tension.

The organisms can be obtained more regularly by *splenic puncture*, but the procedure is rarely justifiable.

Urine cultures are positive in about 10% of the cases, when repeated examinations are made.

The organisms have been cultivated from the stools. Amoss and Poston obtained cultures by adding an agglutinating anti-abortus serum to a suspension of the faeces from which the gross particles had been removed. The agglutinated bacteria were then separated by a short centrifugalization and plated out. The organisms have been grown also from local foci in the joints, gall bladder and meninges.

Guinea pig inoculation is also used to obtain the organisms. This animal is not, however, very susceptible to the bovine type. After about 4 weeks the pig is killed, and cultures are made from the spleen, blood, and lymph nodes. Agglutinins can be demonstrated in the serum.

Agglutination tests are relied upon for diagnosis later in the disease. After the fifth day agglutinins appear in the blood in a large majority of the cases. The test is performed, just as in the Widal reaction (see p. 244), with suspensions of caprine, bovine, and porcine strains as antigens. A titer of 1-60 is considered suggestive and 1-100 diagnostic, although it is often much higher. Proagglutinoid zones are common, and a number of dilutions should be set up. A rising titer has added significance. The agglutinins may persist for years after recovery.

Francis and Evans have pointed out that cross agglutinations occur with  $P.\ tularensis$ , and serum from suspected cases of brucellosis should be tested for agglutinins with this organism also. The titer of the serum will usually be higher for the homologous species, or, if both organisms are agglutinated to nearly the same extent, they can be identified by agglutinin absorption tests.

The intradermal injection of the bacteria, or of culture filtrates, gives rise to local and general allergic reactions in cases of brucellosis. Leavell and Amoss believe that this test is of great value in diagnosis when other procedures are negative, and have obtained the best results with heated vaccines made from representative strains.

Treatment with vaccines or sera has not yet been shown to be of value.

## TULARAEMIA

Pasteurella tularensis (Bacterium tularense) McCoy and Chapin, 1911.—This organism has been found in more than 20 forms of wild life,



Fig. 17.—Pasteurella tularensis. Note change from coccus-form to rod-form in a single transfer on culture medium. (Photomicrograph by Major G. R. Callender, M. C., U. S. A., by courtesy of Surgeon E. Francis, U. S. P. H. S.)

especially rabbits and hares, and is transmitted to man by contact with the tissues of these animals or by the bites of various infected arthropods. Cases of tularaemia have been reported from all parts of the U. S., and from Japan, Canada, Russia, Norway, and Sweden.

Morphology and cultural characteristics.—P. tularensis is a very small, non-motile, Gram-negative bacillus, from  $0.3\mu$  to  $2.0\mu$  in length. In fresh cultures short bacillary

#### TULARAEMIA

forms occur; later coccoid forms predominate. Occasionally polar granules may be seen. In tissue smears the organisms appear to be surrounded by capsular material. They stain readily with ammonium oxalate crystal violet in smears from cultures and tissues, but Giemsa's stain is preferable for tissue sections.

They do not grow on plain media. They were cultivated first by McCoy and Chapin on a medium of coagulated hen's egg yolk. Francis found that the presence of cystine

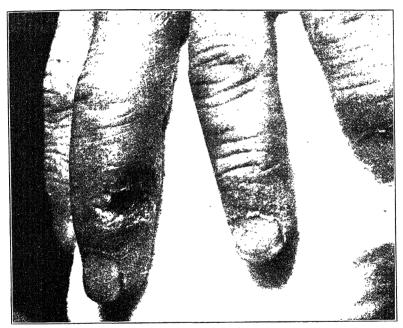


Fig. 18.—Ulcer of finger nineteen days after onset in a market man who dressed rabbits. (Brown and Hunter.)

in the medium is necessary for their growth and has devised a blood glucose cystine agar which is the most satisfactory medium for isolation and culture. (See section on media.) He has found that cultures kept for 12 years are still as exacting in their cystine requirements as when first isolated. On this medium colonies appear about the third day and are smooth, rounded, and opaque. The organisms are strictly aerobic and grow best at 37°C. The optimum reaction of the medium is pH 6.8 to 7.3. On account of their small size they will pass through some of the coarser bacterial filters. The organism causes fermentation with the production of acid but no gas in dextrose, glycerin, mannose, maltose, dextrin and levulose.

These organisms are readily killed by heat (10 minutes at 56°C.); hence thoroughly cooked meat is not infectious. Francis has kept them alive and virulent in glycerinated

guinea pig spleen tissue at -14°C. for 10 years. They have been found to remain virulent in the dried faeces of infected bed bugs for 25 days. No toxins have been demonstrated.

The disease in animals.—Wild rabbits, ground squirrels, wild rats and mice, opossums, and other animals have been found naturally infected. It is transmitted from one animal to another by the bites of various arthropods (flies, ticks, lice, and fleas), and probably also by direct contact. The disease takes the form of a rapidly fatal septicaemia with glandular enlargement and focal necroses in the spleen, liver, lymph nodes, bone marrow, and lungs. The bacteria can be found in large numbers in certain of the tissues.





Fig. 19.—Guinea pig spleens, showing acute (left) and subacute (right) lesions of tularaemia. (Francis.) (Army Medical Museum No. 40325.)

Transmission.—The disease in man is acquired most commonly by handling the tissues of infected wild rabbits or other animals. The bacteria gain entrance through an abrasion in the skin or through the conjunctivae. It is believed that they may also penetrate unbroken skin. Other cases are acquired by the bite of the horse fly (Chrysops discalis) or of the wood ticks (Dermacentor andersoni and Dermacentor variabilis) which have become infective by feeding upon diseased rodents. Francis has found that in ticks the organisms may be harbored for long periods within the body cells and coelomic fluid as well as in the lumen of the gut. Hereditary transmission has been demonstrated from infected ticks to their eggs, larvae, and nymphs. Thirty-nine laboratory infections of man have occured in twelve laboratories among those who autopsied infected animals. Ingestion of insufficiently cooked wild rabbit meat caused 20 cases of which 12 died.

Clinically, tularaemia is characterized by an irregular fever lasting 2 or 3 weeks or longer, with (usually) an initial local ulceration or a conjunctivitis, and with swelling

and, occasionally, suppuration of neighboring lymph nodes. The mortality is not high—under 5% in recognized cases, but the constitutional disturbances may be marked and prolonged. Some cases are ambulant throughout. Four general types of the disease have been described.

- r. Ulceroglandular.—In this type there is an ulcerating papular lesion at the site of inoculation with enlargement and sometimes suppuration of the regional lymph nodes. This is the commonest form of the disease. This type also includes cases of the pulmonary and the meningeal forms, nearly all of which present evidence of infection through the skin.
- Oculoglandular.—The initial infection is a conjunctivitis, and the neighboring nodes are enlarged.
- 3. Glandular.—There is no primary lesion, but the group of glands is affected which drains the site of infection.
- 4. Typhoid.—Pyrexia occurs, but there is no local lesion or glandular enlargement. This type has been particularly common among laboratory workers.

Pulmonary manifestations.—Bronchitis, pneumonia, a clinical picture of chronic tuberculosis, and pleural effusions have developed in a good many cases. Pleural fluid was found to agglutinate P. tularensis in 3 cases and to yield cultures of the organism in 3 other cases. In two fatal cases the organism was recovered from the consolidated lung at autopsy. In 3 cases the organism was recovered from sputum (during life).

Immunity.—An attack of the disease confers a lasting immunity. However, one laboratory investigator who recovered from the disease 17 years ago has since had four transient local re-infections on a finger at different times without systemic disturbance.<sup>1</sup>

Laboratory diagnosis.—P. tularensis has been isolated from the blood early in the disease in a few instances, and from the local lesions and glands, but direct cultivation is difficult and impracticable for diagnosis. The most satisfactory method is animal inoculation. Material from the primary lesion, regional lymph nodes, or blood is injected subcutaneously or intraperitoneally into guinea pigs, rabbits, or mice, which usually succumb within a week. At autopsy the animal shows a haemorrhagic oedema at the site of inoculation, caseation of the lymph nodes, and small necrotic foci in the spleen and liver. Smears from these tissues, stained with crystal violet, usually show numerous organisms, and they may be cultivated from the lesions and from the heart's blood. Blood glucose cystine agar is inoculated with the blood or with small pieces of liver and spleen. Growth appears about the third day. Subcultures grow luxuriantly on this medium.

Infection can be produced in guinea pigs by cutaneous inoculation as in the case of the plague bacillus. Agglutinins develop in the infected animals if they live 10 to 14 days.

<sup>&</sup>lt;sup>1</sup> Personal communication.

Agglutination tests are relied upon chiefly for diagnosis after the first week. A titer of 1-80 or over is considered diagnostic, particularly if the titer rises as the disease progresses. Titers as high as 1-5000 have occurred, and a positive agglutination may persist for months or even years after recovery.

In the National Institute of Health the antigen is prepared by washing off a 48 hour growth on blood glucose cystine agar with a small amount of saline containing 0.3 to 0.5% formalin. The suspension is then thrown down in the centrifuge, and the bacterial sediment is taken up in saline containing 0.3% formalin. This concentrated stock suspension is diluted at the time of use with saline. This suspension may be obtained from the National Institute of Health at Washington, D. C. Non-virulent cultures are suitable for growing antigen.

Francis and Evans have pointed out that there is an antigenic relationship between *P. tularensis*, and *Brucella abortus* and *melitensis*, and that some tularaemia sera contain group agglutinins for the latter organisms. Such tularaemia sera agglutinate *P. tularensis* more quickly and to a higher titer than they agglutinate the Brucella. If tularensis and brucella organisms are agglutinated out to the same titer by a serum, the practical lesson is that a serum suspected of either tularaemia or undulant fever should be tested against tularensis and against one of the brucella.

## GRAM-NEGATIVE BACILLI GROWING ON ORDINARY MEDIA

#### PLAGUE

Pasteurella pestis (B. pestis) Kitasato, Yersin, 1894.—This organism is a member of the group of bacteria which cause the haemorrhagic septicaemias (Pasteurelloses) of various animals. Plague is primarily an epizootic disease of rats, but in some localities ground squirrels and other rodents have been shown to be the source of human infections. This was the terrifying "black death" of the fourteenth century which is believed to have killed 25 million people.

Morphology.—In smears from the tissues P. pestis is seen as a small coccobacillus (1.5 by 0.5 $\mu$ ) with rounded ends, occurring singly or in short chains of two or three. It is non-motile and non-spore-bearing. It is Gram-negative and stains easily with any of the ordinary stains. When it is lightly stained, there is a characteristic bipolar staining which can be demonstrated better by fixing films with absolute alcohol than by heat. Smears from a young culture show typically small, fairly slender rods. In old cultures, and particularly in cultures on agar to which from 3 to 5% of sodium chloride has been added, remarkable involution or degeneration forms which stain feebly are seen: swollen coccoid, root-shaped, or sausage-shaped forms, ranging from 3 to  $12\mu$  in length, and resembling moulds or yeasts rather than bacteria. This peculiarity in morphology on salt agar is characteristic and of great value in identification. In tissue and in cultures grown at 37°C. a gelatinous capsule can be demonstrated. According to Schütze (1932) this envelope does not develop in cultures grown at 20°C., and only meagerly at intermediate temperatures,

PLAGUE

Cultural characteristics.—P. pestis grows readily on the ordinary plain media. It is aerobic, and, unlike most pathogenic bacteria, grows best at a temperature of about 30°C. On agar plates the colonies are minute and transparent the first day, but by the end of the second day they become larger, opaque, greyish, and coherent. In older

cultures there is so much difference in the size and appearance of the colonies that the culture appears to be contaminated. Litmus milk is acidified slightly. A slight acidity is produced in glucose but not in lactose broth. Ordinary broth cultures show a powdery or granular deposit at the bottom of the tube, and a hanging drop preparation from such a culture may show chains of bacilli resembling streptococci. If cultures are made in broth flasks containing drops of oil on the surface, a characteristic stalactite growth is observed. The culture grows downward from the under surface of the oil drops as powdery threads. These are very fragile, and since the slightest jar breaks them, it is difficult to obtain this cultural characteristic. Plague bacilli are readily

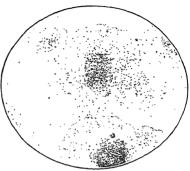


Fig. 20.—Colonies of plague bacilli 48 hours old. (Kolle and Wassermann.)

killed by the ordinary disinfectants and by sunlight, but will withstand drying for at least a month. The virulence of these organisms varies greatly both under experimental conditions and in nature.

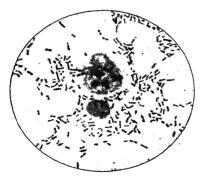


Fig. 21.—Pest bacilli from spleen of a rat. (Kolle and Wassermann.)

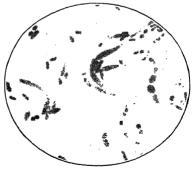


Fig. 22.—Pest bacillus involution forms produced by growing on 3 % salt agar. (Kolle and Wassermann.)

The disease in animals.—Plague is a natural disease of rats, and McCoy has found it in ground squirrels in this country. Other rodents may be infected, and it has been suggested that the Siberian marmot or tarbagan (Arctomys bobac) may have been responsible for plague epidemics in the past. The disease is spread among rats by the

bites of rat fleas from infected animals. A chronic form of rat plague has been described which may be a factor in perpetuating the disease.

Rats dead of the natural disease show characteristic gross pathological lesions which, according to McCoy, are diagnostic. The most characteristic feature is the appearance of the lymph glands. There is a marked haemorrhagic periglandular infiltration with caseation and often haemorrhage in the gland. The subcutaneous tissues are congested and the pleural cavities filled with fluid. The liver is usually yellowish, (occasionally normal) in color, and sprinkled with small greyish white granules which give the organ a stippled appearance as if dusted over with pepper. The spleen is swollen and congested, and often shows similar areas. The bacilli are present in large numbers in the blood, spleen, lymph glands, and other organs. If the carcass is decomposed, they may be isolated by inoculating a rat or guinea pig subcutaneously or intranasally, or by rubbing the material on the shaven skin.

The disease in man.—There are two chief types of plague in man—bubonic and pneumonic. In bubonic plague there may be a local lesion at the site of the bite, or this may be negligible. The regional glands become enlarged and exquisitely tender. In most of the cases the inguinal glands are involved primarily, although in those which McCov traced to ground squirrels the axillary glands were most often affected. Later secondary buboes develop, and suppuration may occur. There is usually a transient bacteriaemia during the first three days of the disease. In the later stages there is often a true septicaemia. In pneumonic plague there is a fulminating and invariably fatal bronchopneumonia or lobar pneumonia with great toxaemia. The sputum is bloody and contains great numbers of the bacilli. The blood may contain them in such numbers (up to 500,000 per cc.) that they can be seen in smears (Strong and Teague). In septicaemic plague there is a blood infection without localized lesions. A cellulocutaneous plague is also differentiated, in which the infection is located in the skin and subcutaneous tissues. The term pestis minor is sometimes applied to mild cases with glandular involvement but with only a slight constitutional reaction, in contrast to the typical disease, pestis major.

Transmission.—Investigations in India determined definitely that bubonic plague is spread from rats and other rodents to man by the bite of the flea, Xenopsylla cheopis. In Europe and the United States Ceratophyllus fasciatus is the common rat flea, and it as well as some other species of fleas (but probably not X. ashia) may transmit the disease. Fleas suck blood from infected rats, and it has been shown that plague bacilli may develop in the oesophagus in such numbers as to cause obstruction. When the flea next bites there is a regurgitation from the oesophagus, which carries the plague bacilli directly into the bite wound. The faeces of the flea are also infectious, and inoculation may occur through the skin by rubbing or scratching the bite. During epidemics the disease is spread from person to person by other fleas and by other biting insects. It has been shown that bed-bugs may also transmit plague.

Pneumonic plague is spread by direct contact (droplet infection). Strong and Teague, studying the epidemic in Manchuria in 1910-11, exposed plates in front of the mouths of these patients during a cough, and recovered plague bacilli in nearly 50% of these cultures. It has been noted that the type of plague which follows wild rodent epidemics (sylvatic plague), such as occurred in ground squirrels in California, is generally pneumonic. It has been suggested that there may be some biological difference between the viruses of bubonic and pneumonic plague.

Laboratory diagnosis.—The plague bacilli can be demonstrated in smears and cultures from the material aspirated from a bubo. In the later stages when softening begins they are less numerous or absent. In pneumonic plague the bacilli are found in the sputum. In septicaemic cases and in very early cases (within three days of the onset) they may be grown in blood cultures. The bipolar staining, the characteristic involution forms, and the stalactite formation in broth are differentiating features of particular value. For precise identification of the organisms agglutination tests with an immune serum should be carried out.

A guinea pig or other rodent should be inoculated with the material to demonstrate the characteristic lesions. The material may be emulsified and injected subcutaneously with a syringe, or a bit of the material may be put in a pocket under the skin. Animals may also be infected by rubbing the material over the shaven skin or by applying it to the nasal mucosa. This method is especially applicable to contaminated material such as sputum. The ability to infect an animal through the unbroken skin has been regarded as a crucial test of the plague bacillus, but other organisms, notably *P. tularensis*, may be inoculated in this way.

Immunization.—One attack of plague is believed to confer immunity. During the disease agglutinins develop both in man and animals. Their demonstration is of some diagnostic value, although the titer is usually low. Agglutination in a dilution of r-5 or r-10 is considered positive.

Prophylactic vaccination has been used extensively, especially by Haffkine in India, and both the incidence and the mortality of the disease have been greatly reduced. At present the vaccine is prepared by emulsifying the growth on agar slants and killing the organism by heat, as with other vaccines. Flu and others have used vaccines made from cultures dissolved with bacteriophage. Strong vaccinated a number of individuals with living cultures which had been rendered avirulent, but this method has not been used widely on account of its possible danger.

Schütze (1932) identified two antigenic constituents in plague bacilli, a somatic antigen and one occurring in the gelatinous capsule which is only present in cultures grown at 37°C. The former antigen is heat-stable and the latter heat-labile at 100°C. He found that vaccines containing both antigens were much more effective in immunizing animals than those having only the somatic antigen.

Therapeutic sera prepared by the injection of horses or cattle with living organisms have been used by Yersin and others. Good results have been reported except in the pneumonic cases when large doses are given intravenously (Naidu and Mackie, 1931).

### Pasteurelloses in Animals

Other haemorrhagic septicaemias in various animals are due to organisms differing only slightly in cultural characteristics. They may be differentiated by their specific infectiousness and by serological tests. Of these the P. pseudo-tuberculosis rodentium (Corynebacterium pseudotuberculosis, Bergey) is closely related to P. pestis antigenically

(the somatic antigen is common to both species, according to Schütze), but it has been shown by Arkwright to be motile under certain conditions. It is the cause of a fatal septicaemia in guinea pigs, and occasionally causes confusion in animal experimentation. It is not distinctly pathogenic for white rats. P. aviseptica of chicken cholera, P. suiseptica of swine plague, and P. cuniculicida of rabbit septicaemia and "snuffles" belong to this group. P. tularensis produces somewhat similar lesions in guinea pigs, but rats are much less susceptible to this species than to the plague bacillus. They may be differentiated by serological tests.

Klebsiella pneumoniae (B. pneumoniae, B. mucosus capsulatus) Friedländer, 1882.—This organism is responsible for about 5% of the cases of pneumonia, both lobar and lobular. These cases are severe, and usually fatal. It is also often associated with suppurative processes in the sinuses and middle ear. In children it may cause tonsillitis. It has been isolated from the blood, and from the spinal fluid in cases of meningitis. Flu and others have reported organisms of this genus in cells of the granulomatous tissues in granuloma venereum.

Morphology.—The Friedländer bacillus is a short, thick, rod, varying in size, but averaging I  $\times$  2.5 $\mu$ , non-motile and Gram-negative. It has a large capsule which can be demonstrated in smears from pathological material, such as sputum or animal exudates. The bacilli often occur in diplo-form, or in short chains or groups, surrounded by a continuous capsule.

Cultural characteristics.—The colonies on agar are large, semi-transparent, whitish, and very viscid, later tending to become confluent. On potato it shows a thick, sticky growth containing gas bubbles. The distinctive cultural characteristic is the "nail" appearance in a gelatin stab. The growth at the surface is heaped up like a round headed nail, the line of puncture resembling the shaft of the nail. The gelatin is not liquefied. Many of the carbohydrates are fermented with the production of acid and gas, but most of the strains do not produce gas in lactose. No indol is formed. These latter characteristics help to differentiate it from some of the colon bacilli with which it may be confused. If in doubt, inoculate a mouse at the root of the tail. Death from septicaemia occurs within 2 days, and the encapsulated bacilli can be found in the sticky peritoneal exudate, and in the blood and organs.

The Friedländer bacillus resembles closely the Acrobacter aerogenes, but their relationship is not clear.

Scrological types.—Three distinct serological types, A, B, C, have been differentiated by Julianelle among strains from various sources. He designates other apparently unrelated strains not falling into these types as group X. Type specificity is dependent upon the carbohydrate haptene in the capsule. The carbohydrate of the type B Friedländer bacillus resembles chemically and serologically that of the type II pneumococcus, and a Friedländer antiserum protects mice against this pneumococcus and vice versa. The specific soluble substance has been demonstrated in the urine of patients with a Friedländer pneumonia, by precipitin tests with immune sera.

Julianelle dissociated smooth, encapsulated, virulent organisms into rough, non-encapsulated, non-virulent forms. The latter contain only the group antigen in the

cell body, and immune sera prepared with them will agglutinate all organisms of this species.

Klebsiella rhinoscleromatis.—This bacillus is associated with rhinoscleroma, a granulomatous inflammation of the mucous membrane of the nose, throat and mouth. In sections of the nodules of the nasal mucosa are seen large cells with crescentic nuclei (Mikulicz cells) which may be packed with these bacilli. The organism resembles Friedländer's bacillus very closely, except that it produces no gas from glucose, and does not ferment lactose.

Klebsiella ozaenae.—This bacillus has been found in cases of ozaena, but its etiological relationship is questionable. It resembles Friedländer's bacillus closely, but produces no gas in glucose.

Klebsiella granulomatis. —(See p. 625.)

## THE INTESTINAL GROUP OF BACILLI

These bacteria are all morphologically similar, aerobic, Gram-negative, non-spore-bearing bacilli which do not liquefy gelatin or solidified serum. This group includes both parasites and saprophytes which inhabit the intestinal tract of normal animals. They have many characteristics in common, and serological as well as cultural methods may be required for differentiation. There is a great deal of group agglutination among the different species, and agglutinin absorption tests and other special procedures may be necessary for identification. In some cases the antigenic constituents must be studied separately.

The group is subdivided into:

- 1. The Eberthella or typhoid group.
- 2. The Salmonella group. This includes the S. schottmülleri (B. paratyphosus B.), S. paratyphi (B. paratyphosus A.), S. enteritidis (Gärtner), S. aertrycke—associated with typhoid-like fevers and food poisoning, and S. morgani, S. suipestifer, and others.
- 3. The Shigella or dysentery group. This includes several different species— S. dysenteriae (Shiga), S. paradysenteriae (Flexner types) and other related forms.
- 4. The *Escherichia* or colon group. This includes a large number of different species—*E. coli*, *E. communior*, etc.

Cultural procedures for the differentiation of these organisms are summarized in the table on the back cover.

## THE TYPHOID BACILLUS

TYPHOID FEVER

Eberthella typhosa (B. typhosus) Eberth, 1880; Gaffky, 1884.—This organism is the cause of typhoid fever.

Morphology and cultural characteristics.—The typhoid bacillus is from 1 to  $3\mu$  by 0.6 $\mu$ . It is more slender and more actively motile than members of the colon group. It grows readily on plain media, but its growth is more delicate and transparent than that of the colon bacillus. Occasionally the colonies resemble grapevine leaves. Its

growth on potato is less abundant than that of *E. coli*, and it does not discolor the potato. Broth is diffusely clouded. Litmus milk changes at first to reddish-violet, later, blue; it is not coagulated. The bacillus ferments dextrose and mannite, forming acid but not gas, and does not ferment lactose or saccharose. It does not produce indol. For its action on carbohydrates see table, on back cover. The reaction observed when inoculated by streak and stab on Russell's double-sugar medium is of great value in differentiation. The butt of the tube becomes acid without gas formation, whereas no change occurs on the slant. On plates of Endo or Teague medium the colonies remain colorless because they cannot ferment the lactose.

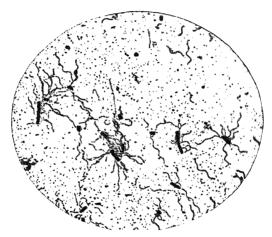


Fig. 23.—Bacillus of typhoid fever, stained by Löffler's method to show flagella. (×1000.) (Williams.)

Variation of the organisms of this group is of the greatest practical importance. Variation in the physical characteristics of the colonies (smooth and rough) occurs, and motile and non-motile varieties of either S or R forms are encountered. Arkwright describes the following chief variants of the typhoid bacillus: (1) smooth, motile; (2) smooth, non-motile; (3) rough, motile; and (4) rough, non-motile. These types differ antigenically.

There are two different types of antigen in the smooth forms.

1. The flagellar or H antigen derived from the flagella, which produce the large-flake agglutination with its antiserum. This antigen is heatlabile at 80° to 100°C., and is destroyed by 50% alcohol. An alcoholized suspension of the organisms, therefore, contains only the following O antigen.

2. The somatic or O antigen, contained in the body of the organism, which produces a small-flake or granular agglutination. This antigen is heat-stable, resists boiling, and is destroyed by formalin. A formolized suspension, therefore, contains only the H antigen.

The dissociation from the smooth to the rough type is associated with some change in the somatic O antigen, the variant being expressed by the symbol  $\emptyset$  (or R), whereas the flagellar (H) antigen is the same in both smooth and rough motile forms.

Grinnell believes that the somatic antigen is fundamentally similar in both smooth and rough forms, and that the antigenic differences between O and  $\emptyset$  are due to an associated carbohydrate haptene, or specific soluble substance. This, he thinks, either is diminished in amount in the rough forms, or is replaced by a different carbohydrate. According to his conception, therefore, the antigens of the four variant types are the:

- (), somatic antigen common to all forms,
- II. flagellar antigen of the two motile types.
- S. carbohydrate haptene of the smooth forms.
- R. possible carbohydrate peculiar to the rough forms.

In the study of the antigenic relationships of this group of organisms it is necessary to investigate these antigens separately, in addition to making the ordinary agglutinin absorption tests, since some have a common somatic antigen with different flagellar antigens, and others a similar flagellar antigen, but different somatic antigen. For instance, the O antigen of the typhoid bacillus is similar to that of S. enteritidis, while their H antigens are quite different. Likewise the H antigen of E. typhosa appears to be the same as the H antigen of the Stanley type of Salmonella in its specific phase. The problem is relatively simple in the case of the typhoid bacillus, since the H antigen is monophasic and specific—that is, not variable in its serological reactions, as is that of some of the paratyphoid bacilli.

Pathogenicity.—Typhoid fever can not be produced in animals except perhaps in the chimpanzee. It is possible, however, by intravenous injection of the bacilli to produce in rabbits a local infection of the gall bladder which may persist for weeks—a chronic carrier state. Large doss of living or dead bacilli may kill an animal through the action of the endotoxin. Mice can be infected by intraperitoneal injections of organisms suspended in mucin. See Prophylactic Vaccination, below.

Typhoid fever.—The organisms enter the body through the alimentary tract. During the period of incubation they multiply in the lymphoid tissue of the intestine, particularly in the Peyer's patches, and later invade the lymphatics. The adjacent lymphatic glands, and in particular the spleen, become involved. After a time, which is approximately the period of incubation, they become so abundant that they are carried over into the general circulation, and a transient bacteriaemia results. When this happens bacteriolysis of the organisms occurs, with liberation of the endotoxins and the development of symptoms. Since the bacilli do not multiply to any extent in the blood itself, the condition must be regarded as a bacteriaemia rather than a septicaemia. This invasion of the blood stream occurs early in the disease in practically all cases. The bacilli usually reappear in the blood at the onset of a relapse.

As a result of the formation of antibodies the typhoid bacilli disappear from the blood, and become localized in other areas. They are present in the gall bladder, prob-

ably in all cases. They may produce no symptoms, or a cholecystitis may result. Bile is a favorable culture medium, and they may persist here for years. They have been cultivated from the center of gall stones many years after the original attack.

After the first week the bacilli increase in number in the stools, and are more easily found in cultures. This increase in number is due not to multiplication within the lumen of the intestine, but to discharge into the intestine of infected bile, and probably to ulceration of the Peyer's patches. Frequently they may persist for years in the stools, and the person becomes a chronic carrier.

During this period of the disease the bacilli are often (25% to 30%) found in the urine. Transient periods of bacilluria are frequent. They may cause no lesions, or suppurative conditions in the kidney, pyelitis, or cystitis may follow. In either case they may remain in the urine for years.

In cases with an associated bronchitis or bronchopneumonia the bacilli can be found in the sputum.

Localized lesions elsewhere due to the typhoid bacillus alone or in combination with some other organism may occur late in the disease—periostitis, osteomyelitis, or deep abscesses. The bacilli have been found in the cerebrospinal fluid in cases with meningitis. They have been demonstrated in the lymph spaces of the rose spots.

After the first or second week demonstrable antibodies develop in the blood—bacteriolysins, opsonins, precipitins and agglutinins. The demonstration of agglutinins in the blood, the Widal reaction, is the most important diagnostic procedure after the bacteriaemia has subsided. Agglutinins may not appear until convalescence, however, and fail to develop in about 5% of the cases.

Carriers.—Although most typhoid fever patients become bacteria-free after about 3 months, a certain number (2 to 5%) develop into chronic carriers and may remain so for many years. Most of these carriers harbor the organisms in the gall bladder, some in the intestinal tract only, and some in the urinary passages. The bacilli have been found in the faeces of contacts who did not contract the disease. Dissemination of the bacilli by these carriers is the great factor in perpetuating the disease. Large epidemics have been caused by pollution of the water supply. Contamination of food, especially milk, by carriers or by flies has resulted in outbreaks. The chlorination of water supplies and better methods of sewage disposal have greatly lessened the incidence of typhoid. The detection of carriers, especially among individuals who handle food, is of great importance. The treatment of carriers seems to be of little use. In cases in which the gall bladder is shown to be the source of the infection, cholecystectomy offers the best chance of relief from the carrier state.

Laboratory diagnosis. Blood cultures.—During the first week of the disease typhoid bacilli can be isolated from the blood in about 90% of the cases. Positive cultures are obtained in from 70% to 60% in the second and third weeks, and after that time the percentage falls further. For methods of culturing the blood see chapter on blood cultures. The organism should be identified culturally, and by agglutination with typhoid, paratyphoid A and paratyphoid B antisera. Occasionally a strain is not agglutinable when first isolated, but becomes so after a few subcultures.

Stool cultures are occasionally positive early in the disease, but after the first week the typhoid bacillus may be isolated in most of the cases. The material is streaked over Endo, bismuth sulphite or Teague or brilliant green plates, as described in the chapter on Faeces. When it is practicable to pass a duodenal tube, the typhoid bacillus can be isolated more readily by making cultures from the bile in the same way. Identification by serological as well as cultural methods is necessary.

Urine cultures are positive in about 25% of the cases after the second week. The urine should be centrifuged, and the sediment cultured, or broth flasks heavily inoculated. Plates of special media should be used unless the urine is obtained with aseptic precautions.

No patient should be released from isolation until cultures from the urine and faeces after catharsis, and preferably from the bile also, are negative. These methods are also used to detect carriers.

Widal test.—After the second or third week the Widal test is the chief diagnostic aid. This should be repeated at intervals in order to detect any increase in the titer of the agglutinins. The technique of the microscopic and macroscopic agglutination test, and methods of preparing the antigens are described in the chapter on Immunity and Hypersensitiveness. Cultures of known agglutinability must be used. In making the macroscopic tests it is customary for the sake of safety to use a suspension which has been killed by the addition of 0.1% of formalin. Such a suspension contains the H antigen only, but for clinical purposes it will usually suffice. Occasionally, however, only the O agglutinin develops in the body, and the Widal will not be positive unless a living culture or an alcoholized emulsion is used. Since typhoid and the paratyphoid fevers may be indistinguishable clinically, it is necessary to test the agglutination of these strains also with the serum. Some group agglutination of these organisms is very common in typhoid in the higher concentrations of the serum, but it is less marked than that with the typhoid bacillus, and is unusual in dilutions of over 1-40.

In individuals who have not received typhoid vaccine, agglutination with a 1-50 dilution of serum justifies a strong suspicion of typhoid fever, which is confirmed if the titer rises as the disease progresses. Agglutination in a dilution of 1-100 or more is practically diagnostic of active typhoid infection. Carriers may show some slight agglutination, but there is no important change in the titer of the serum on successive examinations.

If, however, the individual has previously received typhoid vaccine, difficulties arise in the interpretation of a positive agglutination test. Within a few days after vaccina-

tion the serum will usually show a high titer of specific agglutinins. This is followed by a fall in titer, at first rapid and later very gradual, so that over a short period the titer is practically unchanged. If such an individual develops typhoid fever the titer of the serum will increase as the disease progresses. This, however, may occur also in infections other than the typhoid fevers, as for instance in infections due to the pyogenic cocci or other bacteria—the so-called "anamestic reaction." One can only conclude that the results of the Widal reaction in vaccinated individuals must be interpreted with the greatest caution, even when the titer is high and rises with the progress of the disease. The reaction is of no value for the detection of carriers.

Alropin test.—Manson-Bahr regards the Marris atropin test as of great value in the diagnosis of the enteric group of fevers. A hypodermic injection of  $\frac{1}{50}$  grain of atropin sulphate is given. If the case is typhoid or paratyphoid, the pulse rate is practically uninfluenced by the injection. In normal individuals and in other infections the pulse rate drops at first but after 10 or 15 minutes rises above the initial pulse rate by more than 15 beats and usually by 30 or 40 beats per minute during the period of 25 to 50 minutes following the injection. This test is rarely used in the U. S.

Prophylactic vaccination.—The value of prophylactic vaccination as originally introduced by Wright has been amply demonstrated. The vaccine is prepared in the usual way and killed by heating to 53°C.

For many years most of the typhoid vaccine used in this country and in England was made from a single culture, "Rawlings," isolated by Wright in 1900. Grinnell (1932) has found that some cultures of this strain have become dissociated partially or completely, and have lost their virulence for mice. Vaccines from these cultures did not protect mice from infection with recently isolated, smooth strains as well as did vaccines made from a smooth strain, although vaccination with the Rawlings strain still produced somatic and flagellar agglutinins. He concludes, therefore, that virulent smooth strains should be substituted for the Rawlings strain for the production of vaccine, and that the demonstration of its ability to produce H and O agglutinins is not an adequate measure of its immunizing power.

This question has been investigated by Colonel Siler and his associates of the Army Medical Corps in an attempt to increase the protective properties of the typhoid vaccine used in the U. S. Army. In their experiments seven strains were tested, three of high virulence and four of low virulence. In cross-immunity tests mice vaccinated with virulent organisms were protected to a much higher degree than those receiving vaccines of low virulence. Of the virulent strains tested, one (No. 58) isolated from a chronic carrier of many years duration was the most effective, and this is now used in the preparation of the new vaccine. This is a smooth variant with high virulence and immunizing power, as tested by the production of active immunity in mice and protective power for mice of the serum of vaccinated individuals.

The employment of agglutination titer as a measure of immunity is unsatisfactory, because there is not a close parallelism between them. There is a marked difference in titer in different individuals after vaccination, and in a given individual, after reaching a peak in about 30 days, the agglutinin titer falls rapidly. Immunity may exist in the absence of agglutinins.

The more satisfactory mouse protection test was, therefore, used. It was found that with typhoid bacilli of low virulence such large doses had to be used to kill the controls that the treated mice were overwhelmed with foreign protein before resistance and immunity could be brought into play. If a virulent culture was used, and if the

bacilli were suspended in 6% mucin and injected intraperitoneally, the M.L.D. ranged from 10 to 1000 organisms for the special strain of mice used, instead of 100,000,000 to 1,000,000,000, if suspended in Ringer's solution. In testing the protective power of the serum before vaccination a standard dose of culture of 10,000 living virulent organism was injected. If all the mice succumbed within 72 hours it was assumed that the man was not immune to typhoid fever.

Two methods of preparing vaccine were used; in one, the vaccine was heat killed and preserved with 0.25% tricresol; in the other, formalinized (0.1%). No distinct difference in the immunizing power of these vaccines was demonstrated. Vaccines were prepared by both methods from a culture of low virulence (the Rawlings intermediate strain which has been used in the Army for years) and from a highly virulent strain (No. 58).

The subjects, who had not had typhoid fever and had not been vaccinated previously, were divided into four groups. Each received three subcutaneous injections of one of these vaccines. The first dose was 500,000,000, and the two subsequent doses 1,000,000 each. A statistical study of the results obtained showed a materially higher degree of protective power for mice when human immune serum following vaccination with virulent organisms was used than with organisms of low virulence.

Formerly a triple vaccine was used in the Army made up of 500,000,000 typhoid bacilli, 250,000,000 paratyphoid A bacilli and 250,000,000 paratyphoid B bacilli in 1 cc. of vaccine. The vaccine now in use contains only typhoid bacilli, 1,000,000,000 per 1 cc. The first dose is 0.5 cc., the second and third 1 cc. each. The vaccine is inoculated subcutaneously at intervals of one week.

From 1917 to 1924 triple vaccine was used in the Navy. Good statistical evidence showed that paratyphoid infections, other than cases of "food poisoning," were rare among naval personnel before paratyphoid vaccine was used. With the vaccine containing *E. typhosa* alone a larger amount of typhoid antigen can be used without undue risk of severe reactions with presumably greater protection against typhoid fever, which is the infection against which protection is principally required.

The U. S. Naval personnel receive two courses of three inoculations at intervals of 7 to 10 days, four years apart, of a vaccine containing approximately one billion typhoid bacilli per cc. The first dose is 0.5 cc., the second and third 1 cc. each.

There may be a slight rise in temperature (rarely above ror°F.) with headache and malaise in about half of those inoculated.

Agglutinins appear in the blood within a few days. By the end of a month titers of 1-640 or 1-1280 are frequently reached. The immunity produced is believed to last for from 2 to 5 years, and there is some evidence that it may last much longer.

Neither vaccines nor therapeutic sera have proved to be of any value in the treatment of typhoid fever.

## SALMONELLA OR PARATYPHOID—ENTERITIDIS GROUP

This is a heterogeneous group of organisms, some of which cause various gastrointestinal disorders, and occasionally a disease resembling typhoid fever. This group includes *S. paratyphi* (paratyphoid A), *S. schottmiilleri* (paratyphoid B), *S. enteritidis* (Gärtner), and *S. aertrycke*.

and the less important but closely allied organisms, S. suipestifer, and S. morgani.

These organisms are alike morphologically and culturally on ordinary media. They may be differentiated roughly from the colon group by their inability to ferment lactose and saccharose; and from the typhoid and dysentery groups by their ability to ferment dextrose and mannite with gas production. The individual types react alike on a Russell tube, producing acid and gas in the butt, and no change on the surface of the slant. For other biochemical reactions see table on back cover.

#### PARATYPHOID FEVER AND FOOD POISONING

Paratyphoid bacilli (Achard and Bensaude, 1896; Schottmüller, 1901).—Paratyphoid fever, which bears a close resemblance to typhoid fever clinically, may be caused by S. paratyphi (A) or S. schottmülleri (B).

Salmonella paratyphi (paratyphoid A) is distinguished from other members of the Salmonella group by its inability to ferment xylose, and to produce a brownish discoloration on lead acetate agar. This group is serologically relatively homogeneous.

Salmonella schottmülleri (paratyphoid B) produces acid and gas from xylose, discolors lead acetate agar, and eventually renders milk strongly alkaline. Strains in this group vary in their antigenic properties, and may be very difficult to classify.

Both organisms may cause a clinical picture indistinguishable from that of typhoid fever, although the symptoms are apt to be milder. They are present in the blood in the early stages, and later appear in the faeces, and sometimes in the urine. Agglutinins develop, and the diagnosis may be made by agglutination and agglutinin absorption tests. A great many clinical types of paratyphoid infections occur, since there is, as a rule, less tendency for the bacteria to localize than for typhoid bacilli. Among these have been noted a dysenteric type, a nephritic type, a rheumatic type, and an influenzal type. Some cases of infectious jaundice have been attributed to paratyphoid infection, and also various local infections such as pyelitis. Paratyphoid B may cause symptoms resembling those of meat poisoning. It is more pathogenic for animals than is the typhoid bacillus. The development of antibodies in man and in animals is much less marked than that which occurs in typhoid. Infection is transmitted in the same ways as is typhoid, and some of the cases become chronic carriers.

Immunization with vaccines is discussed in the section on typhoid fever.

Laboratory diagnosis.—The same methods are used as in typhoid fever. Precise identification of the organisms is sometimes difficult even by agglutinin absorption tests, especially in the case of paratyphoid B, which may be diphasic. The flagellar antigen of a given strain may occur in two phases: a specific phase, in which it is agglutinated only by a strictly homologous antiserum; and a non-specific or group phase, in which it is also agglutinated by antisera produced by the injection of the H antigen of other (heterologous) types. For exact identification of the organism it is necessary to test the agglutinibility of the flagellar and somatic antigens separately. If the flagellar antigen is in the group phase, it is essential that the culture be dissociated into the specific phase, if necessary by growing it in media containing a group antiserum antagonistic to the heterologous elements of the group antigen.

Antigenic structure.—The antigens of the Salmonella group have been studied in detail, especially by White and by Kauffmann, and the classifi-

cation proposed by the latter investigator was recommended for general adoption by the Salmonella subcommittee of the International Society of Microbiology, 1934. Thus far more than 40 types differing in their antigenic structure have been described. The type name is usually that of the locality at which the strain was first isolated.

The O antigens, of which 13 have been distinguished, are designated by arbitrarily chosen Roman numerals. The flagellar antigens in the specific phase, of which 28 have been identified, are designated by letters (a to z,

## ANTIGENIC STRUCTURE OF SALMONELLA

		Flagellar H antigen		
Type of organism	Somatic O antigen	Specific phase	Group phase	
S. paratyphi (Paratyphoid A) Senftenberg	I, II I, III	a gs		
S. schottmülleri (Paratyphoid B) aertrycke (typhi-murium) Stanley Reading	IV, V	b i d eh	I, 2 I, 2, 3 I, 2 I, 4, 5	
Brandenburg		enlv		
S. suipestifer, American type suipestifer, European type	VI, VII		I, 3, 4, 5 I, 3, 4, 5	
ThompsonPotsdam		k enlv	1, 3, 4, 5	
Newport Newport, var. Puerto Rico Newport, var. kottbus	VI, VIII	eh  eh	I, 2, 3 I, 2, 3 I, 3, 4, 5	
E. typhosa S. enteritidis. enteritidis, var. Dublin enteritidis, var. Moscow. sendai. Panama	IX	d gom gp goq a lv	I, 4, 5 I, 3, 4, 5	
	S. paratyphi (Paratyphoid A) Senftenberg  S. schottmülleri (Paratyphoid B) aertrycke (typhi-murium) Stanley Reading  Brandenburg  S. suipestifer, American type suipestifer, European type  Thompson Potsdam Newport Newport, var. Puerto Rico Newport, var. kottbus  E. typhosa S. enteritidis enteritidis, var. Dublin enteritidis, var. Moscow sendai	S. paratyphi (Paratyphoid A) I, II Senftenberg I, III  S. schottmülleri (Paratyphoid B) aertrycke (typhi-murium) IV, V Stanley Reading  Brandenburg  S. suipestifer, American type suipestifer, European type VI, VII Thompson Potsdam Newport Newport, var. Puerto Rico Newport, var. kottbus  E. typhosa S. enteritidis enteritidis, var. Dublin enteritidis, var. Moscow sendai Panama	Type of organism  Somatic O antigen Specific phase  S. paratyphi (Paratyphoid A) I, II Senftenberg I, III gs  S. schottmülleri (Paratyphoid B) aertrycke (typhi-murium) Stanley Reading Brandenburg  S. suipestifer, American type suipestifer, European type VI, VII  Thompson Potsdam Newport Newport, var. Puerto Rico Newport, var. Rottbus  E. typhosa S. enteritidis enteritidis, var. Dublin enteritidis, var. Moscow IX goq sendai a Panama I, III a Specific phase  IV, V i Stanley d k enlv VI, VIII III III Specific phase	

z<sub>2</sub>, etc.), and those in the group phase by Arabic numerals (r to 6). The antigenic structure of a strain is usually determined by subjecting suitable suspensions of the organism to various immune sera from which all or most of the agglutinins, except that corresponding to the antigen to be investigated, have been removed by absorption with suitably chosen strains. Each type has usually 2 or 3 different O antigens; in the specific phase, r to 4 H antigens, and in the group phase, 2 to 4 (other) H antigens. To identify a given type the culture must be in a smooth state, the organism must be examined in both the group phase and the specific phase, and it may be necessary to demonstrate the presence of 8 different antigens.

An organism may be assumed to be in the group phase if it is agglutinated by an H-agglutinating serum for *S. suipestifer*, European type, since the latter occurs only in the group phase.

The table on page 121 illustrates the complexity of structure and the apparently haphazard way in which the different O and H antigens are combined in some of the types. (For a full discussion see Topley & Wilson, Bacteriology, 1937.)

Salmonella enteriticis (B. enteritidis) Gärtner, 1888.—This organism has been isolated frequently from cases of gastroenteritis caused by the ingestion of meat from diseased animals, or even of food contaminated by contact with the infected meat. The infection may be spread by the unclean handling of food, by flies, or even by the contamination of food with the faeces of mice or rats.

It closely resembles the paratyphoid B bacillus in its cultural reactions, but can be differentiated from it by its ability to produce acid in tartrate media and by agglutination tests with immune serum.

In man S. enteritidis causes an acute gastroenteritis with symptoms of intoxication. Since Salmonella have been isolated from only 20 to 30% of the cases of food poisoning, Savage (1920) suggested that the symptoms in some cases may be due to endotoxins formed in the meat before ingestion. If the amount of toxin ingested is great, symptoms occur shortly after ingestion. If the amount of toxin is small, the symptoms may be delayed for one or two days. This toxin, unlike that of Cl. bolulinum, is not destroyed by boiling. The Gärtner bacillus has been isolated in pure culture from the faeces in cases with high fever and marked intestinal derangement with fluid stools containing considerable blood. It is very pathogenic for laboratory animals, producing a haemorrhagic enteritis and at times a septicaemia.

Salmonella aertrycke (B. aertrycke).—This organism frequently causes a similar gastroenteritis. It resembles the paratyphoid B bacillus even more closely than S. enteritidis, and is difficult to differentiate serologically, even by agglutinin absorption tests. Both are diphasic, but in the specific phase they possess different flagellar antigens. S. aertrycke (identical

with B. typhi-murium, B. pestis caviae and B. psittacosis) is highly pathogenic for many laboratory animals and causes serious epidemics, especially among mice and guinea pigs.

S. suipestifer (B. suipestifer).—This organism was isolated by Salmon and Smith from swine with hog cholera, and was believed to be the etiological agent. It is now known, however, that the disease is caused by a filtrable virus and that, although this organism is constantly present and may be isolated from the blood, it is only a secondary invader. It has been reported as the cause of epidemics of food poisoning in man, although in some of these cases it was probably confused with S. aertrycke. It has also been isolated from sporadic cases of a severe general infection in man (most frequently in children) which resembles typhoid fever clinically. (Reviewed by Harvey, 1937.) Identification of this organism requires precise agglutination and agglutinin-absorption tests. The H antigen of the European type is monophasic (group phase), whereas that of the American type is diphasic (see table). According to Kuttner and Zepp it can also be differentiated by its inability to ferment arabinose, trehalose and inositol.

Salmonella morgani has been reported as the cause of certain cases of mild enteritis. It produces a very slight amount of gas in glucose only and produces indol. It does not cause any primary acidity in litmus milk.

Other closely related organisms with slight antigenic differences have been described in various epidemics.

	Botulism	Food infections		
Cause	Botulinus toxin.	Bacilli of the salmonella group.		
Fever	Not characteristic; temperature usually subnormal.	Characteristic; acute.		
Occurring	Mainly in winter.	Mainly in summer.		
Associated with	Preserved foods.	Fresh foods, or freshly con- taminated foods, usually meat or milk.		
Condition of bowels	Constipation; rarely diarrhoea.	Diarrhoea; offensive.		
Visual disturbances	Double vision; ptosis of lids.	Absent.		
Abdominal pain	Absent.	Present.		
Onset	Usually gradual.	Sudden.		
Incubation period	Variable, usually from twelve hours to several days.	Short, usually from six to twelve hours.		
Throat	Swallowing difficult.	Normal.		
Treatment	Antitoxin.	Systemic.		
Mortality	From 60 to 70%.	From 1 to 2%.		

Laboratory diagnosis in cases of food poisoning due to these organisms depends chiefly upon their isolation from the stools by plating methods. Occasionally they may be demonstrated in blood cultures. During convalescence agglutinins may be formed which can be tested against known strains of these bacteria. Cultures from the infected food should be made when possible.

"Ptomaine" poisoning.—This term is based upon a misconception. The split products of protein putrefaction have not been demonstrated to have a toxic effect when ingested. This diagnosis has often been applied to disturbances resulting from infection with Salmonella, dysentery or colon bacilli. Botulism must also be considered. Anaphylactic reactions to certain food stuffs may cause similar gastrointestinal disturbances.

Burke and May have tabulated the differences in botulism and food infections as shown on page 123.

## THE SHIGELLA OR DYSENTERY GROUP

### BACILLARY DYSENTERY

Shigella dysenteriae (Shiga, 1898) and paradysenteriae.—Dysentery bacilli cause an acute inflammation of the mucous membrane of the large intestine, and occasionally the lower part of the ileum, with the appearance of mucus, blood, and pus in the stools. The bacilli are present in the intestinal tract, and at times in the mesenteric glands, but do not invade the blood stream or appear in the urine.

Morphology and cultural characteristics.—The dysentery bacilli occur, as a rule, singly or in pairs, and do not form threads or filaments. They are somewhat plumper than typhoid bacilli. In their cultural characteristics they resemble the typhoid bacillus, but can be distinguished from them by their lack of motility, as well as by agglutination reactions (see table on back cover).

Types.—There are two main types of dysentery bacilli:

- 1. Shigella dysenteriae (Shiga-Kruse type) which produces no acid in mannite media, and does not form indol.
- 2. Shigella paradysenteriae (B. flexneri) (Flexner, Hiss (Y), Strong, and others) which produces acid in mannite media and forms indol.

The Shiga type is homogeneous in its antigen content and may be differentiated from other types serologically, although there is often some group agglutination with other types. In its growth it forms a potent exotoxin as well as an endotoxin. Intravenous injection of the bacilli, or of their toxins, into rabbits produces a haemorrhagic enteritis, followed later by paralysis of the hind legs. An antiserum can be prepared which

possesses marked antitoxic and antibacterial properties, and is of therapeutic value if given early.

An organism which resembles the Shiga type in its inability to ferment mannite' but which produces indol and ferments rhamnose, is known as the Schmitz bacillus (S. ambigua).

The group which ferments mannite is composed of a number of types—S. paradysenteriae, types Flexner, Hiss, Strong, and others, which are antigenically heterogeneous; S. sonnei, S. dispar, etc. They produce an endotoxin, but not the soluble exotoxin which is characteristic of the Shiga bacillus. Clinically the toxaemia in dysenteries due to these types is less than that in the Shiga type.

In addition there are other atypical types such as the Sonne bacillus, (S. sonnei) which produces acid in mannite, and also a slow fermentation of lactose, and forms no indol. This represents a distinct serological type, and has caused epidemics of dysentery in this country and in Europe.

S. dispar resembles S. sonnei except that it (usually) ferments xylose and forms indol. Serologically it constitutes a heterogeneous group. It is probably non-pathogenic.

Dysentery bacilli may be differentiated provisionally by the following cultural reactions:

Shigella	Dex- trose	Man- nitol	Maltose	Sucrose	Indol
Dysenteriae (Shiga).				0	
Ambigua (Schmitz)				0	
Paradysenteriae:					
Hiss type (Y)				0	
Flexner type				0	
Strong type			0	+	
Sonnei			+	+	
Dispar			+		

Recent work has shown that the types of S. paradysenteriae are variable in their fermentative reactions, and that groups based on these properties do not correspond to those based on serological reactions. The latter classification is, therefore, preferable.

Andrewes and Inman have shown that strains of this group possess, in different proportions, at least 4 (or 5) different antigenic constituents which they designate as V, W, X, (Y), and Z, and that in the individual types one or another of these preponderates. In the Hiss (Y) strain these antigens are usually present in relatively balanced proportions, and antisera prepared with this strain will agglutinate most of the other members of the group. For the production of therapeutic sera a number of strains of different antigenic properties are used. This serum is often combined with an anti-Shiga serum for immediate use in cases of dysentery.

Laboratory diagnosis.—The appearance of the stools in acute bacillary dysentery is characteristic. There is usually an absence of faecal material,

and the bulk of the stool is composed of clear or turbid, amber colored, serous fluid in which float curled masses of white mucus flecked with bright red blood. In amoebic dysentery the typical stool contains faecal material and blood. It has been shown that the microscopic appearances of the stools of these two types of dysentery differ. The cell exudate in bacillary dysentery is abundant, and is composed largely of polymorphonuclear leukocytes which show toxic degenerative changes, and frequently large macrophages. In amoebic dysentery there are comparatively few cells in the exudate and mononuclear cells are more numerous than polymorphonuclears unless marked secondary infection is present (see Figs. 85 and 86). Warm stage preparations should be searched for amoebae. If the gross and microscopic examinations suggest bacillary dysentery, and if no amoebae are found, it is considered desirable to give the polyvalent therapeutic serum without waiting for a bacteriological report, since to be effective, serum must be administered early.

During the first week the dysentery bacilli may be cultivated from the stools without difficulty. They are often present in large numbers, or even in practically pure culture in the first 2 or 3 days, but diminish rapidly in number as the faecal character of the stools returns. Isolation from chronic cases and from carriers is very difficult and uncertain.

The mucus is spread on lactose litmus agar or Teague plates, on which the colonies are colorless and resemble those of the typhoid bacillus. After identifying the organism by its cultural characteristics, final identification is made by agglutinating the organism obtained with a Shiga bacillus antiserum, and also with a polyvalent serum of the Flexner group. The Sonne type must be agglutinated with its specific antiserum. Agglutinin absorption tests are usually necessary to determine the precise type to which the strain belongs. Occasionally a strain when first isolated is not agglutinable, and the test must be repeated after subculturing. The organism isolated should be tested later for agglutination with the patient's serum. During epidemics the serum of convalescents may be used instead of immune sera, if these are not available, to identify strains obtained from the stools.

The development of agglutinins in the patient's blood occurs only after 1 or 2 weeks, and hence is of relatively little value in diagnosis. The test is carried out in the same manner as the Widal reaction with representative strains of dysentery bacilli. The titer of the serum is usually not high. Agglutination in a dilution of 1-50 is generally considered as positive for the Shiga bacillus, and 1-150 for the paradysentery types. The agglutinability of the known strains in normal serum must be tested as a control.

Vaccination.—Vaccines of the Shiga bacillus made in the ordinary way are very toxic for man as well as for animals, and must be combined with the antitoxin or treated with formalin to reduce their toxicity. Those of the paradysentery group may be used in small doses without detoxification. They have been used with some success in prophylaxis intramuscularly, and also by oral administration. Reports of their value in the treatment of acute and chronic cases are contradictory.

Alcaligines faecalis (B. fecalis alkaligenes).—This organism is a frequent inhabitant of the intestinal tract.

It does not ferment any of the sugars, and in milk cultures there is a progressive alkalinity, with the liberation of a little ammonia. It is strikingly aerobic.

This bacillus does not seem to have any effect on animals. It has been isolated from the blood of a few cases which resembled typhoid, and was agglutinated by their sera (1-50). It has been under suspicion in some cases of diarrhoea in children.

## ESCHERICHIA OR COLON GROUP

Escherichia coli (B. coli).—This species includes a large number of types which differ in minor characteristics. The most important member of the group, E. coli, was isolated by Escherich in 1886 from the faeces of infants. It is the common inhabitant of the intestinal tract of man and other animals, and its presence in water in an index of sewage contamination.

Morphology and cultural characteristics.—E. coli is a Gram-negative, rod-shaped or oval organism with rounded ends, averaging about 0.6 by 2.5 $\mu$  in size. It is motile, but less actively so than the typhoid bacillus. The degree of motility varies with different strains and with the same strain under different cultural conditions. It is most marked in young cultures (under 12 hours). The bacillus grows luxuriantly on all ordinary media. In broth it produces a uniform turbidity, with, at times, a surface scum, and often a somewhat foetid odor. It acidifies and coagulates litmus milk, and ferments most of the ordinary sugars, including glucose and lactose, with the production of acid and gas (see table on back cover). Potato is discolored to a brownish color. Gelatin is not liquefied. It produces indol. On Endo plates the colonies are bright red and develop a metallic scum after about 36 hours. Inoculated by streak and stab on Russell's tubes, the whole medium is acidified and gas develops in the butt.

Special tests are used to differentiate the colon bacillus from allied species (described in the section on Bacteriology of Water). With typical strains the Voges-Proskauer reaction is negative, and the methyl red test is positive. The colon bacilli are unable to utilize citrate as a source of carbon, as does .1. aerogenes, and do not grow in the synthetic citrate medium.

Pathogenicity.—The colon bacilli appear in the intestinal tract shortly after birth, and continue to be present in large numbers throughout life. It is probable that they exert some protective action in the intestinal tract, but they are capable of producing a variety of infections elsewhere in the body. They are particularly prone to cause urinary tract infections, and in young children a colon bacillus pyelitis is a frequent cause of fever. They often cause infections of the gall bladder and biliary passages, peritonitis from perforation of the gastrointestinal tract (often in association with other organisms), and suppurative lesions in any part of the body. Colon bacillus infections are more common in the tropics than in temperate climates, and many cases of prolonged fever are due to a colon bacillus bacteriaemia. Owing to the tendency of these bacilli to post mortem or agonal invasion of the blood stream, a positive culture at this time has little or no significance.

Normal serum agglutinates the colon bacillus in dilutions of I-10 or I-20, and this titer may be increased after typhoid infections because of the production of group agglutinins.

Escherichia communior (B. coli communior) is a closely related organism which was given this name because of its supposed greater frequency in faeces. It is similar in all respects to E. coli except that it ferments saccharose with the production of acid and gas.

Aerobacter aerogenes (B. lactis aerogenes).—This organism is found in the intestinal tract of man and other animals. Like E. communior it produces gas in glucose, lactose, and saccharose broth, but does not ferment dulcite. It is, as a rule, distinctly nonmotile, and often seems to be surrounded by capsular material. The colonies are large and very viscid. Except for its greater fermentative activities it might be confused with the Friedländer bacillus. Unlike the colon bacillus it gives a positive Voges-Proskauer reaction, and is methyl red-negative. It frequently grows in the synthetic citrate medium. Gelatin is not liquefied. It has been reported as a cause of cystitis.

## GELATIN-LIQUEFYING FAECAL BACTERIA GROUP

This is a group of putrefactive organisms which are closely related to the colon bacilli, but which liquefy gelatin.

Aerobacter cloacae (B. cloacae).—Isolated first from sewage by Jordan. It is, as a rule, a rapid liquefier of gelatin, although not as active as the proteus group. In its motility and in its reactions with litmus milk and the sugars it resembles the colon bacillus. The Voges-Proskauer reaction is positive, the methyl red test negative, and, like the A. aerogenes, it gives the citrate-utilization reaction.

Proteus vulgaris.—This organism is often encountered in plates made from water contaminated with sewage. It may cause localized infections. Certain strains are agglutinated by the serum of patients with typhus fever and other Rickettsial infections.

Morphology and cultural characteristics.—The bacillus is very motile, long and slender, tending to form filaments. It is Gram-negative. The spreading growth on solid media is characteristic of the group. From the central colony irregular, translucent streamers spread out and coalesce until the whole surface of the medium is covered. Glucose, maltose, and generally saccharose are fermented, but not lactose. Litmus milk is coagulated and alkalinized. Later the litmus is reduced and the clot digested and the medium becomes a dirty yellowish-brown fluid. Indol is rarely produced. The cultures generally have a putrefactive odor. Gelatin is rapidly liquefied, and some strains liquefy coagulated blood serum.

Pathogenicity.—Proteus vulgaris is common in putrefying organic material. It has been reported as the cause of acute food poisoning, and has been found in the blood in these cases. It may cause various suppurative processes, such as otitis media, peritonitis, cystitis and pyelitis, usually in association with other organisms.

Variation.—Non-motile forms of Proteus vulgaris occur which grow in round, thick colonies without the characteristic spreading. Weil and Felix applied the term O (ohne Hauch) to their strains of this variety in

contrast to the motile spreading types which they designated as H (Hauch). Since their original work, the letter H has come to be applied to the flagellar antigen and antibodies of various motile bacteria, and the letter O to the somatic antigens and antibodies of the non-motile forms.

Weil-Felix reaction.—Weil and Felix isolated from the urine of three typhus fever patients two strains of Proteus vulgaris which they designated as X2 and X19. These strains are non-motile and non-spreading (ohne Hauch), and, therefore, are dissociated into the O form. These strains are agglutinated by the sera of patients with typhus fever and other Rickettsial diseases, although they are neither the causative agents nor secondary invaders. The reaction is, therefore, heterologous and non-specific, but it is an almost constant phenomenon, is not present in other conditions, and is of great practical value in diagnosis. The test is described in detail in the section on typhus fever.

Castaneda and Zia (1933) ascribe this reaction to a common antigenic factor in *Proleus X*19 and the *Rickettsia prowazeki*. Immunization of animals with either organism produces agglutinins for both. *Proleus X*19 serum absorbed with the homologous organism loses all its agglutinins, but when absorbed with the *Rickettsia* it is still capable of agglutinating the *Proteus. Rickettsia* (or typhus) serum absorbed with the *Proteus X*19 is still capable of agglutinating the *Rickettsia*, but when absorbed with *Rickettsia* the agglutinins for both are removed. The agglutinin in the typhus serum which acts on the *Proteus X*19, therefore, corresponds to a minor agglutinin.

### THE GROUP OF LACTOBACILLI

This is a group of long, non-sporing, Gram-positive bacilli which are capable of resisting a considerable amount of acid in their environment. These organisms occur in the intestinal tract, especially of nursing infants, where they may be the predominant type of organism. They are also found in the mouth, the vagina (Döderlein's bacillus), and in milk.

Lactobacillus acidophilus.—This is a normal inhabitant of the large intestine. The bacilli are large and variable in size, and may occur in short chains or filaments. They are non-motile. There is considerable pleomorphism, and polar bodies may be seen with Neisser's stain. They sometimes resemble diphtheroids in appearance. They are aerobes and facultative anaerobes, and when cultivated in this way they resemble the L. bifidus. They rarely grow below 22°, and the optimum temperature is between 37° and 40°C. Glucose and lactose are fermented with the formation of acid but not gas. Maltose is usually fermented also. Milk is slowly curdled. Original cultures are best made in 1% or 2% glucose, 0.5% acetic acid broth. Frequent transfers are necessary, since viability decreases rapidly. In man the feeding of lactose or dextrin usually results in an increase in their relative number in the intestinal tract, owing to the production of acid which renders the environment less favorable for other organisms, especially putrefactive bacteria. This is probably facilitated by the administration of

large amounts of young cultures in milk or broth. Alteration of the intestinal flora in this way has been attempted in the therapy of some forms of intestinal disorders.

Lactobacillus bifidus, so named on account of the frequency with which it is arranged in a Y shape in cultures, is found in the stools of nursing infants. It is usually described as a strict anaerobe, but it is closely related to, if not identical with, L. acidophilus.

Lactobacillus bulgaricus, originally isolated from Yoghurt, a fermented milk, resembles the acidophilus bacillus, but does not ferment maltose. Attempts have been made to change the intestinal flora by the ingestion of this organisms, but it is now believed that it cannot be implanted in man.

Lactobacillus boas-oppleri, probably a variety of L. acidophilus, is found in the stomach contents in conditions in which there is gastric stasis and the normal acidity is reduced or absent. Döderlein's bacillus is found in the vagina, and is probably identical with the acidophilus bacillus. Lactobacillus odontolyticus (B. acidophilus odontolyticus) has been described in connection with dental caries. It is frequently present in these areas, and is possibly one factor in their development.

#### CHROMOGENIC BACILLI

These are identified by the color of their colonies on agar. The *Pseudomonas aeruginosa* is the most important one of them in medicine, but the *Serratia marcescens* is also of interest.

Pseudomonas aeruginosa (B. pyocyaneus) Gessard, 1882.—This organism is frequently termed the bacillus of green or blue pus. It is widely distributed in water and air, on the skin, and in the upper respiratory tract, and is often isolated from faeces. It is associated sometimes with other pyogenic organisms in abdominal abscesses. It is probably more pathogenic than has been supposed, and is sometimes the sole cause of cystitis, otitis media, mastoiditis, enteritis in children, and, occasionally, even of septicaemia.

Morphology and cultural characteristics.—This bacillus is small, slender  $(2.5 \times 0.5 \mu)$ , motile, and Gram-negative. It grows readily at room or body temperature. Growth on agar is abundant, moist, and greenish, and a bright green color is diffused through the agar. Gelatin is rapidly liquefied. On potato the colonies are a deep olive-green to dirty brown. Blood serum is digested, and the pitted surface shows a reddish brown color. No gas is produced from any of the carbohydrates, and acid is formed only from glucose. No indol is produced.

In the presence of oxygen, pigments are formed which have been separated into two main types: (r) Pyocyanin, a blue-green pigment, soluble in both water and chloroform, and (2) Fluorescin, a yellowish green, fluorescent pigment, soluble in water but not in chloroform. A red pigment, pyorubin, has also been described.

A large number of variants have been described—R forms, non-motile, and non-pigment producing strains; and an encapsulated variety has been reported.

Toxins.—In addition to an endotoxin, the pyocyaneus bacillus produces a soluble toxin similar to diphtheria and tetanus toxin, but different in that it will withstand a temperature of 100°C. for a short time.

The fact that the union between toxin and antitoxin is only of a binding, neutralizing nature is shown by taking a mixture of pyocyaneus toxin and antitoxin, which is innocuous, and heating it to 100°C. This destroys the antitoxin, but does not injure the toxin, which retains its original toxicity. On the other hand the toxins of diphtheria and tetanus are less stable than the corresponding antitoxins, and, therefore, heating would destroy the toxins first.

The B. fluorescens liquefaciens of water seems to be simply a strain of P. aeruginosa. Pyocyaneus bacilli produce a proteolytic ferment, pyocyanase, which is antibacterial and which has been used therapeutically in diphtheria as a local application. An haemolysin has also been demonstrated in broth cultures.

Serratia marcescens (B. prodigiosus).—This is a very small motile coccobacillus. It is Gram-negative. The colonies on agar or other solid media show a rich red pigment which develops only at room temperature. The S. marcescens is frequently found on food-stuffs, especially bread, where it has been mistaken for blood. It liquefies gelatin rapidly and produces a diffuse turbidity in broth. It is probable that B. indicus and B. kiliensis are strains of S. marcescens.

On account of its size and visibility it is often used to test for leaks in filters. It should be held back by Berkefeld V filters.

A violet chromogen, Chromobacterium violaceum (B. violaceus), has been described under many names. It has been found in water.

#### CHAPTER VI

# STUDY AND IDENTIFICATION OF BACTERIA—SPIRILLA. KEY AND NOTES

KEY to recognition of gelatin-liquefying, motile and Gram-negative spiral or comma-shaped organisms.

- I. Give the nitroso-indol reaction with sulphuric acid within twenty-four hours.
  - (a) Very pathogenic for pigeons.
    - (1) Vibrio metchnikovi (Spirillum metchnikovi). Liquefies gelatin about twice as rapidly as the cholera. Gives bubble appearance at top of stab. Produces an acute enteritis in fowls. Injection of culture into pectoral muscles of pigeons produces a fatal septicaemia. Not pathogenic for man.
  - (b) Scarcely pathogenic for pigeons.
    - (2) Vibrio comma (Spirillum cholerae).
- II. Do not give the nitroso-indol reaction (cholera red) with sulphuric acid alone in twenty-four hours, and furthermore, especially in the case of Denecke's spirillum, the cholera red reaction may be negative after prolonged cultivation.
  - (a) Produce an abundant moist cream-colored growth on potato at room temperature.
    - (1) Vibrio proteus (Finkler and Prior's spirillum). Liquefaction of gelatin very rapid. No air-bubble appearance at top of liquefied area. Cultures have foul odor. Milk coagulated. Thicker and somewhat larger spirillum than that of cholera. Isolated from cholera nostras.
  - (b) Scanty growth or none at all on potato at room temperature. Only a moderate yellowish growth when incubated at about body temperature.
    - (2) Vibrio tyrogenus (Spirillum tyrogenum; Denecke's spirillum). Does not liquefy gelatin so rapidly as that of Finkler-Prior. Milk not coagulated. Thinner and smaller spirillum than that of cholera.

Note.—Non-motile, non-liquefying and Gram-positive spirilla have also been described. There is also a large group of phosphorescent spirilla.

#### CHOLERA

Vibrio comma (Spirillum cholerae) Koch, 1884.—This organism is the cause of Asiatic cholera.

Morphology.—Typically, the organism is a small, comma-shaped rod, 1.5 by  $3\mu$ . It frequently occurs in S shapes, owing to the attachment of a pair of organisms at their ends, and in cultures long threads showing a somewhat spiral appearance may be seen. In smears made from bits of mucus and cellular debris in the faeces, they often resemble fish swimming parallel to one another in a stream. After artificial cultivation, and

occasionally in freshly isolated cultures, rod forms, coccoid, and club-shaped involution forms are frequent. Ohno found that their development depends in part upon the reaction of the medium, and suggested that transfers be made on media of varying pH to obtain the characteristic vibrio morphology. There is a single, long, terminal flagellum which imparts to the organism a very active "scintillating" or "darting" motility. It stains easily by ordinary methods and is Gram-negative.

Cultural characteristics.—The Vibrio comma is strictly aerobic and grows readily upon ordinary culture media. The optimum reaction is pH 8.0-9.0. Growth is inhibited by a moderate acidity, but will occur on media sufficiently alkaline to inhibit other species of bacteria. This tolerance for alkalinity facilitates their isolation from the faeces by special media. On agar the colonies are translucent, bluish grey, resembling somewhat those of the typhoid bacillus. On gelatin plates they are more characteristic, and appear after 24 hours as small, highly refractile, whitish colonies which,

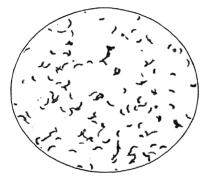


Fig. 24.—Cholera vibrios. (Kolle and Wassermann.)



Fig. 25.—Involution forms of the vibrio of cholera. (Van Ermengem.)

under the low power, have a granular center with spinose margins, and a surrounding zone of liquefaction. In gelatin stabs a turnip-shaped area of liquefaction appears at the top of the puncture—the air-bubble appearance. Coagulated blood serum is liquefied. Litmus milk is (usually) not acidified nor coagulated. On alkaline potato the growth is whitish and later changes to a brownish yellow or pinkish color. In broth or in Dunham's peptone solution growth is rapid and luxuriant, especially at the surface, and a pellicle is formed. In the latter medium indol is produced, and the nitrates are reduced to nitrites. The addition of a few drops of concentrated H<sub>2</sub>SO<sub>4</sub> alone will bring out a red color due to the formation of nitroso-indol—the cholera-red reaction (see p. 842). The ability to ferment carbohydrates varies somewhat with different strains. Some produce acid from glucose, saccharose, maltose, and mannite. Lactose is occasionally fermented after 10 to 12 days. Gas is not produced from any of the sugars.

The typical cholera vibrio does not produce haemolysis on blood media, although after several days growth there may be some chemical alteration or digestion of the medium around the colony which simulates a zone of haemolysis. Cooked blood

medium is cleared in the same way. If a filtrate from a broth culture is added to a suspension of red blood cells, no haemolysis occurs. An exception to this rule is the El Tor vibrio which was isolated from cases of fatal diarrhoea in pilgrims at El Tor. This organism is actively haemolytic, yet is agglutinated by cholera immune serum.

Serological relationships.—Recent studies, especially those of Gardner and Venkatraman (1935), have shown that the antigenic structure of V. comma and related vibrios is very complex. The true cholera strains appear to constitute a relatively homogeneous group. There are, however, numerous strains obtained from various other sources which are culturally and biochemically identical with V. comma and possess the same H antigen but which have different O antigens. They are, therefore, agglutinated by ordinary anti-cholera serum which contains both H and O agglutinins. A diagnostic immune serum, therefore, should be prepared from an antigen from which the non-specific H component has been removed. Some of the El Tor vibrios have been found to possess the specific O antigen of the true cholera group, while others are related to it only through the H antigen.

The cholera vibrio is readily dissociated, and a number of variant strains have been produced by various methods of cultivation. According to Linton et al., these changes depend primarily upon a loss or chemical alteration of the specific carbohydrate. In one rough variant he found that both protein and carbohydrate constituents were altered. Experimentally, variant types may be produced also by the action of various types of bacteriophage. These changes are ascribed by Morison (1935) to alterations of the bacterial protein by hydrolysis.

Toxic products.—Kraus believes that the cholera vibrio secretes a true exotoxin in addition to an endotoxin. This exotoxin is formed by the El Tor vibrios also, and an antitoxic serum prepared from either is capable of neutralizing both.

Pathogenicity.—Feeding, or subcutaneous injection, of the cholera vibrio does not usually cause infection in adult animals. Koch, however, produced the disease in guinea pigs by injecting the organisms together with alkali into the stomach, and giving them opiates to inhibit intestinal peristalsis. Intraperitoneal injection usually gives rise to a fatal peritonitis. However, when the organisms are injected into an immunized guinea pig, or when a small amount of immune serum is simultaneously injected, bacteriolysis takes place (Pfeiffer's phenomenon). If material is removed from the peritoneal cavity with a pipette, at intervals of from 10 to 60 minutes, the cholera vibrios will be found to have lost their motility, and to have become granular and degenerated. Other vibrios are unchanged. The reaction may be demonstrated in a pipette if fresh serum is used. Agglutinating and bacteriolytic sera of high titer can be prepared by the immunization of animals.

Pigeons are almost insusceptible to the true cholera vibrio but are readily infected by a closely allied species,  $V.\ metchnikovi$ , which is not pathogenic for man. It is more pathogenic for guinea pigs than the  $V.\ comma$ .

Transmission.—Cholera is endemic in India, and practically every pandemic has been traced to this area. Another endemic focus is believed to exist in China. The disease is spread by contamination of water supplies or food by faeces from a patient, or from a carrier. The organism may live for weeks in stagnant water, and great explosive outbreaks have occurred from polluted water. Ordinarily the vibrios disappear rapidly from the faeces (within 5 or 10 days), but Grieg has shown that the biliary tract is often infected, and that they may persist here for a long period of time. Virulent organisms have been demonstrated also in the faeces of healthy individuals who have

been in contact with cholera cases. On the other hand, epidemiological observations indicate that even typical agglutinable vibrios from the faeces may not be virulent, and

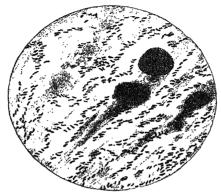


Fig. 26.—Cholera vibrios—"fish in the stream appearance." (After Jochmann from Mayer.)

the possibility of the dissemination of the disease by chronic carriers has been questioned.

The disease in man.—The onset is abrupt after an incubation period of from 2 to 5 days. A profuse diarrhoea occurs, usually without colic or tenesmus. The stools soon lose their faecal character and consist of a watery, slightly opaque fluid containing flocculi of desquamated epithelium and mucus (the characteristic so-called rice-water stools). Similar material is vomited. At this stage muscle cramps are frequent, probably associated with the great loss in chlorides caused by the diarrhoea and vomiting. There is great toxaemia which probably depends in part upon the absorption of the endotoxin. The dehydration is intense, and there is anuria, low systolic pressure, and cyanosis. The subcutaneous tissues are so shrunken that the body has a cadaveric appearance, and the hands look shriveled, the typical so-called "washerwoman's hands." At this stage the blood becomes greatly concentrated, the red blood cells frequently reaching 7 or 8 millions, with a corresponding increase in the leukocytes. The proteins of the blood are concentrated, and the specific gravity is raised. As a result



Vibrio cholera. culture in gelatin two days old. (Fraenkel and Pfeiffer.)

of the anuria and the increased tissue destruction, the non-protein

nitrogen of the blood may reach high figures. Sellards found that there is also an acidosis (due to loss of base through the intestinal tract) and obtained great improvement by giving intravenous injections of sodium bicarbonate solution. Equally good results follow large and repeated injections of hypertonic salt solution, to which glucose may be added. If anuria is relieved, the kidney seems to be able to restore the acid-base equilibrium.

Laboratory diagnosis.—During the acute stage of the disease the vibrios can be demonstrated in large numbers in the rice-water stools in smears and by cultures. They do not penetrate into the submucosa, and are seldom if ever found in the blood stream. Exceptionally they have been isolated from the urine, and Grieg has found them in the lungs in convalescents who developed pneumonia. He believes that they are carried to these areas by the lymphatics. In the later stages the diagnosis may be made by agglutination tests.

The comma-shaped organisms may be recognized in smears from a fleck of mucus stained with a I-IO dilution of carbol fuchsin. In such preparations they frequently show the "fish in a stream" appearance.

They may be isolated by plating out some of the material on Dieudonné's medium, which inhibits the growth of most of the intestinal bacteria. When the organisms are relatively scanty (as in the examination of carriers) their isolation is facilitated by the enrichment method. A tube of alkaline peptone solution (pH 8-9) is inoculated with the faeces. In this medium they multiply rapidly at the surface so that a pure culture may often be obtained by removing a loopful from the top after 3 to 8 hours. Stained smears are made and a hanging drop preparation examined. If characteristic motile vibrios are present, a loopful is spread over a dry agar or Dieudonné plate to obtain a pure culture. If their numbers are relatively scanty, a second tube is inoculated from the first, and examined similarly. After isolation the organism is identified culturally. The cholera-red reaction may sometimes be obtained in the peptone solution culture even in the presence of other organisms, although occasionally a pure culture is necessary for the test.

Final identification of the organism is made by agglutinating it with a cholera immune serum, preferably one prepared with the O antigen only. This may be done macroscopically with a broth culture. If the organism isolated is a true cholera vibrio, agglutination and loss of motility occur quickly. Some strains may be inagglutinable when first isolated, however, as is the case with the typhoid bacillus. The demonstration of Pfeiffer's phenomenon with immune serum is the surest proof that the suspected organism is a true cholera vibrio.

When colonies are present on solid media, it is possible to carry out microscopical agglutination tests by emulsifying the colony directly in a loopful of suitably diluted immune serum (1–500 or 1–1000 of a high-titer serum). A hanging-drop preparation is examined under the high power for agglutination and loss of motility. An emulsion in salt solution is made as a control. Subcultures should be made from the same colonies for further studies.

For isolation of the vibrios from water, 900 cc. is added to 100 cc. of 10% peptone solution containing 5% NaCl. This mixture is distributed into a number of sterile flasks, and after 24 hours the surface growth is examined and subcultured.

Agglutinins appear in the patient's serum about the fourth day and reach a maximum in about 2 weeks. They may be demonstrated by agglutination tests with a known cholera strain. The titer is variable, from 1–100 to 1–1000 or higher. Pfeisfer's phenomenon may be obtained with the serum when the agglutination is not clear-cut.

Immunization.—An attack of cholera confers a lasting immunity. Serum treatment has been tried, but the results have not been uniformly favorable. Prophylactic vaccination was first attempted by Ferran in 1885 and later by Haffkine with live cultures. Kolle first introduced the use of killed cultures, which are employed exclusively at present. These vaccines may be prepared by the same methods as typhoid vaccine. Three doses of 1000 and 2000 million are given at intervals of about a week. Castellani combines them with the triple typhoid vaccine. Strong used an autolysate prepared by killing cultures at 60°C. and allowing them to digest at 37°C. for 3 or 4 days. Besredka's bilivaccine has been tried orally to produce local immunization. Reports have shown that vaccination has been of considerable value during epidemics, both in decreasing the incidence of infection and in reducing the mortality.

In several large series the morbidity among the inoculated has been reduced to from  $\frac{1}{4}$  to  $\frac{1}{2}$  of that among those not inoculated. The mortality among those attacked was also reduced, but to a much less extent (about  $\frac{1}{2}$  in some series).

Various other organisms resembling the true cholera vibrio have been isolated from cholera-like conditions. They differ in minor characteristics and are not agglutinated by an anti-cholera O serum. They have been designated the paracholera vibrios. Five groups have been differentiated, based on differences in their O antigens.

#### CHAPTER VII

## STUDY AND IDENTIFICATION OF SPIROCHAETES

## Spirochaetacea

Classification.—It is still disputed whether the spirochaetes should be considered as protozoa, as suggested by Schaudinn 30 years ago, or whether they are more closely related to the bacteria as was originally believed. At present the latter view is more generally held, and Bergey, in his last Manual of Determinative Bacteriology places them in the class Schizomycetes, order Spirochaetales.

Noguchi first proposed a classification of the spirochaetes based largely upon morphological differences (Fig. 28). The following classification, adopted by Bergey, is based upon that of Noguchi, but uses the name *Borrelia* for the genus which he originally termed *Spironema*.

## Spirochaetacea

- 1. Spirochaeta Ehrenberg, 1833. Large free-living fresh-water and marine forms. Type S. plicatilis ( $500 \times 0.75\mu$ ), cylindrical, with regular spirals  $1.5\mu$  apart. Has an elastic flexible axial filament but no crista or flagella. Not dissolved by bile salts or saponin in 10% solution.
- 2. Saprospira Gross, 1911. Large free-living marine and fresh-water forms; type S. grandis (100  $\times$  0.8 $\mu$ ). Is divided internally into chambers by many transverse septa. Organism disposed in numerous relatively rigid undulating curves. There are no flagella nor is there an undulating membrane (crista).
- 3. Cristispira Gross, 1910. Large spirochaetes parasitic in alimentary tract of oysters and other shell-fish; type C. balbianii, Certes, 1882 (45 to  $90 \times 1.8\mu$  with obtuse ends, cylindrical and composed of 2 to 5 large irregular flexures). Has a distinct and flexible longitudinal crest and an internal chambered structure like Saprospira.
- 4. Borrelia Swellengrebel, 1907. Type B. gallinarum. Various sizes, no axial filament, no crista, or undulating membrane. All disintegrated by 10% saponin and bile salts; 5 to 14µ in length by 0.5µ in width, flexible and snakelike. In man B. recurrentis, B. novyi, B. duttoni, B. berbera, B. carteri, B. vincenti, B. buccalis, B. eugyrata, B. bronchialis, etc. Includes B. anserina of geese, B. theileri and many others.
- 5. Treponema, type T. pallidum Schaudinn, 1905. Shaped like a corkscrew, pointed ends, 8 to  $1.4 \times 0.3\mu$  with from 6 to 12 turns of the spiral. With dark ground appears as silvery delicate corkscrew in motion. Imperfect illumination may show them as dots. Curves rigid while in Borrelia they tend to straighten out. Members of both these genera (German theory) composed of ecto and endoplasm, the former being continued beyond the latter forming the attenuated ends.

6. Leptospira Noguchi, 1917, type L. icterohaemorrhagiae—Inada and Ido, 1914. 7 to 14µ in length, 0.3µ in width, with pointed ends and a spiral amplitude of 0.45µ; one or more gently undulating curves. Terminal filament, axial filament, and undulating membrane absent. Resist 10% saponin but are dissolved by bile salts. Three pathogenic species, L. icterchaemorrhagiae, L. hebdomadis, and L. morsus-muris. Progress by rotary motion with one end hooked.

The first 3 genera do not occur in man and are of no medical significance. The members of the genus *Borrelia* are often referred to as the blood spirochaetes in contra-

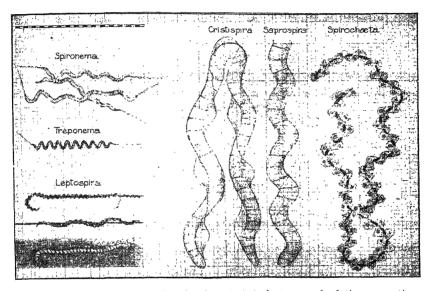


FIG. 28.—Diagram contrasting the characteristic features and relative proportions of Borrelia (Spironema), Treponema, Cristispira, Saprospira, Spirochaeta, and Leptospira. The scale in micra is given in the upper left-hand corner of the figure. (After Noguchi in Journal of Experimental Medicine.)

distinction to the *Treponema* or tissue spirochaetes. Intermediate between these two genera is the *Leptospira* group, the members of which have characteristics in common with both. The *Borrelia vincenti*, however, has not been demonstrated in the blood. The nomenclature of the organism causing rat bite fever is also unsatisfactory. Although included here in the genus *Leptospira* (*Leptospira morsus-muris*), it differs morphologically from other members of the group in the rigidity of its spirals. Unlike other spirochaetes it possesses one or more flagella, and its motility resembles that of the vibrios. On this account many bacteriologists consider that it should be placed in the genus *Spirillum*. Rat bite fever, however, resembles other spirochaetal infections clinically and responds in the same way to therapy with the heavy metals.

Some investigators have described granular forms which they believe represent a stage in the life cycle of the spirochaetes. A filtrate containing such forms has been shown to be infective, but inasmuch as many of the flexible spiral forms may also work their way through bacterial filters, the assumption that the granules are a phase in the life cycle is not thereby proven.

Multiplication of the spirochaetes is now known to be by transverse fission, as in the case of ordinary bacteria, and not by longitudinal fission as was formerly believed.

#### THE BLOOD SPIROCHAETES

Relapsing Fevers.—Relapsing fever is an arthropod-borne, spirochaetal infection which is widely distributed throughout Africa, India,

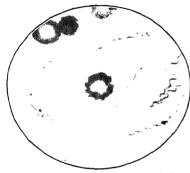


FIG. 29.—Borrelia recurrentis from blood of a man with relapsing fever. (Kolle and Wassermann.)

and eastern European countries. Widespread epidemics have occurred in many other countries including the United States. The disease is characterized by a short febrile period (4 to 10 days) which begins and ends abruptly, and is usually followed after a week or two by a similar but milder paroxysm. In the African "tick fever" there may be as many as 10 such relapses, whereas in the European type of the disease there are rarely more than 2 or 3. With each relapse there is a fresh invasion of spirochaetes into the blood stream, where they

persist until shortly before the crisis. During the remissions the organisms practically disappear from the peripheral blood, but they may be found in large numbers in the hyperplastic spleen in which they are apparently harbored.

There is no significant clinical difference in the disease as it occurs in the various countries. In some regions, however, it is spread by infected lice, and in others by infected ticks. Both louse-borne and tick-borne cases have been reported from the same region in S. America. It has been shown that these spirochaetes can live in the bodies of bed bugs, and it is quite possible that they may also act as vectors.

The spirochaetes causing relapsing fever have been differentiated into several more or less distinct types or species, of which the more clear cut are *B. recurrentis* causing the (louse-borne) European disease, and *B. duttoni* causing African tick fever. Morphologically similar organisms are responsible for various arthropod-borne blood infections in fowls, cattle and other animals.

Borrelia recurrentis (Spirillum obermeieri) Obermeier, 1873.—This is the cause of the relapsing fever occurring in eastern Europe, and some

investigators believe that it is identical with the spirochaete responsible for the disease in India, and in Egypt and the northern part of Africa.

Transmission.—The European infections are transmitted from one individual to another by lice. After biting an infected person, the spirochaetes, which are taken into the alimentary tract of the louse, disappear within a few hours. The insect is apparently harmless for the succeeding 4 or 5 days. At the end of this time spirochaetes reappear in the coelomic fluid of the louse, which then remains infectious for 2 or 3 weeks. It is probable that organisms may be present also in the excreta through which the bite wound may be contaminated. Nicolle and others were able to produce the disease in animals by rubbing the abraded skin with an emulsion of crushed, infected lice after having shown that infection was not produced by their bites or by the injection of their faeces. It is generally accepted, however, that the disease in man is caused by contamination of a bite wound or a scratch by the material from a crushed louse or by its faeces. It is believed that a reservoir of infection exists in certain small rodents.

Morphology.—The Borrelia recurrentis are variable in size, but average from 10 to  $20\mu \times 0.4\mu$ . They are flexible, and have from 4 to 10 open, irregular coils. They have an active corkscrew motility in fresh blood preparations. They are easily stained by the usual bacterial stains and by the Romanowsky blood stains, as well as by silver impregnation methods. Individual organisms may have a beaded appearance, although a majority of them stain uniformly.

Cultivation.—Noguchi succeeded in obtaining cultures of this organism by using a medium containing ascitic fluid, blood, and a bit of fresh, sterile tissue (see p. 853), and incubating them anaerobically. Cultures have since been obtained in media enriched with serum or blood without the addition of fresh tissue.

Animal inoculation.—Monkeys, mice and rats can be infected by subcutaneous inoculation. The disease produced resembles the human infection, and the spirochaetes are demonstrable in large numbers in the blood during the febrile period. Guinea pigs are resistant to infection.

Immunity.—With recovery from the disease a transient immunity develops, and the serum acquires the ability to protect animals from subsequent inoculation. Bactericidal and agglutinating antibodies can be demonstrated. Relapses are frequent, but are ordinarily less severe than the original attack, and may be explained by an inadequate formation of antibodies. The more resistant spirochaetes survive and multiply, and again invade the blood stream. With each relapse the immunity is "stepped up" until recovery is complete. These spirochaetes have been found in the blood of symptomless individuals, and such cases may be of great importance in disseminating the disease.

Laboratory diagnosis.—During the febrile phase of the disease the spirochaetes may be demonstrated in films of the blood stained by one of the ordinary Romanowsky stains, or by dilute carbol fuchsin. They may be

seen in fresh preparations examined by dark-field illumination. They are sometimes found in the afebrile period. If they are not numerous, thick films should be prepared. If they are not found in smears, a mouse should be inoculated with the blood. Within 24 or 48 hours the spirochaetes can be found in the blood of the mouse, if they were present in the inoculum. For demonstrating them in tissue sections silver impregnation methods are used. There is usually a well marked polymorphonuclear leukocytosis in acute cases.

Borrelia duttoni.—This spirochaete is the etiological agent of the relapsing fever prevalent in the western, eastern, and central regions of Africa (African Tick Fever). It is practically indistinguishable from the Borrelia recurrentis morphologically, but it is believed to be a different



Fig. 30.—Borrelia novyi. (Todd.)

species. It is transmitted from one individual to another by a tick (*Ornithodorus moubata*) and possibly, in some cases, by other arthropods.

Spirochaetes are found in the coxal fluid and faeces of the tick, and human infection is caused by contamination of the tick bite by these excreta. The organisms have also been found within the eggs of infected ticks, in the nymphs and even in the third generation. Leishman describes a breaking up of the spirochaetes in the alimentary tract of the tick into small granules which penetrate the Malpighian tubules and the ovary. He regarded these granules as the infecting agents, and suggested that they represent a phase in the life cycle of the spirochaete. This hypothesis, however, has not been definitely substantiated according to Wenyon.

African Tick Fever is similar to the louse-borne European relapsing fever in its clinical manifestations, but multiple relapses are more frequent. The diagnosis is established in the same way. The *Borrelia duttoni* is more virulent for experimental animals than *Borrelia recurrentis*.

Borrelia novyi.—This organism, found in relapsing fever cases in N. America, has been differentiated from other types by immunological reactions. It is apparently transmitted by lice.

Other varieties of spirochaetes have been described—B. carteri in the Indian relapsing fever; B. berbera in Egyptian and N. African cases, B. persica in Persia. These are

#### VINCENT'S ANGINA

not sharply differentiated from other types, however, and it is questionable whether they actually represent separate species.

A relapsing fever has been reported from Texas transmitted by Ornithodorus turicata. This tick inhabits certain caves which harbor various animals. A similar infection in California is transmitted by O. hermsi, and in British Columbia a Dermacentor is supposed to be concerned. Brumpt notes the high degree of hereditary transmission in these ticks, and again suggests that these blood spirochaetes may have been commensals for invertebrates in primitive periods, later infecting vertebrates.

## Fusospirochaetosis

Borrelia vincenti. This organism, together with a fusiform bacillus, is found in small numbers in the mouths of most normal adults, particularly

around the gingival margins and in the tonsillar crypts. They are very numerous in the pseudo-membranous ulcerative inflammations known as Vincent's angina and Vincent's stomatitis, and may complicate other types of ulceration, such as diphtheria, syphilis, or carcinoma.

They are practically always found together, but it is not yet definitely known whether their association is a symbiosis, or whether they represent different forms of the same organism. Some investigators, Tunnicliff, Smith (1932) and others have observed an apparent transformation from one type into another in cultures, while others have



Fig. 31.—Borrelia vincenti. Smear from case of Vincent's angina. (Coplin.)

failed to do so and believe that these two organisms represent distinct species. It is convenient, however, since they are so closely associated in the lesions, to consider them together. Both are obligate anaerobes, and can be grown on enriched artificial media. Cultures have a foctid odor.

Borrelia vincenti is a slender, delicate spirillum with a variable number of shallow and irregular undulations. In dark-field preparations from the lesions they are actively motile. They stain readily with any of the silver impregnation methods (Fontana's stain), and with Giemsa's stain, but may also be demonstrated by slightly overstaining with dilute carbol fuchsin (1-5) or Löffler's methylene blue.

Fusiformis dentium is a coarse, plump fusiform rod. They average from 5 to  $7\mu$  in length, but may vary beyond these limits in the same preparation. They may be straight or slightly curved, and the ends taper to a sharp or dull point. In stained preparations they are characteristically beaded or banded. They can be demonstrated with the ordinary bacterial stains, and are generally Gram-negative. They have been described as non-motile, but in some types a definite and even active motility has been observed.

Classification.—Attempts have been made by various investigators to differentiate the various spirochaetes found in the mouth, but their relationships are still confused. Noguchi described types which he designated as S. mucosum, S. microdentium and S. macrodentium which he regarded as identical with Vincent's spirochaete. Fontana has reported a Leptospira buccalis in a case of noma. Kritchevsky and Séguin report four species, some or all of which may be identical with spirochaetes described by other observers. Three of these, S. dentium, S. acuta and S. tenuis were found commonly in the lesions, and were cultivable; and each one in combination with the fusiform bacilli caused lesions in animals. One species, S. buccalis, they found only occasionally in the normal mouth, and were unable to cultivate it.

Smith (1932) divided the fusiform bacilli into 3 types on the basis of their morphology. Type 1 is the characteristic large, fusiform bacillus commonly seen in Vincent's angina. Type 2 is similar but thinner. Type 3 is smaller, usually straight, and is non-motile. This type was common in the pulmonary infections.

Fusos birochaetosis.—Since the original description by Vincent of these organisms in cases of hospital gangrene, they have been found in a variety of pathological conditions associated with putrid inflammatory ulceration. The commonest sites are around the gums, tonsils, and mucous membrane of the mouth. In these areas they may be confused with diphtheritic, syphilitic, or carcinomatous ulcerations, or they may be present as secondary invaders in these conditions. From here extension may occur into the surrounding tissues, causing extensive necrosis, or into the middle ear, larynx, trachea. and bronchi. Less frequently any of the other mucous membranes may be involvedoesophagus, colon, appendix, and rectum. The external genitals may be the site of fusospirochaetal ulcerations, either as a primary infection, or secondary to syphilitic or chancroidal lesions. Deep phagedenic ulcers may result. Post-operative and other skin wounds may become infected. Many cases of tropical ulcer fall into this category. Finally the bronchi and lungs may be affected, with the development of a putrid bronchitis or bronchopneumonia (particularly post-operative) which is often followed by abscess and gangrene. In these cases the organisms can be found in the sputum and their early recognition is imperative since without appropriate therapy the mortality is high—40% according to Kline and Berger. These organisms are frequently found in pyorrhoeal pockets, and although they have no direct causal relationship to the condition, they undoubtedly aggravate it. Such areas when neglected have been aptly termed "anaerobic incubators." These organisms practically always disappear from the mouth after extraction of all the teeth.

The ulcers on section consist of three distinct layers. The superficial layer contains necrotic cellular material and masses of mouth bacteria with varying numbers of spirochaetes and fusiform bacilli. Below this is a layer of acutely inflamed tissue in which the fusiform bacilli predominate. The deep layer contains masses of spirochaetes which appear to be the actively invasive agents. Streptococci usually are demonstrable throughout the lesion. There is little or no leukocytic infiltration.

Pathogenicity.—The rôle which these organisms play in the production of these conditions is not entirely clear. Practically always other bacteria are found associated with them, and whether they are the primary etiological agent or whether they are present as secondary invaders is disputed. Kritchevsky and Séguin believe them to be primary, and have produced local abscesses in animals and occasionally a generalized spirochaetosis by the injection of pure cultures of spirochaetes and fusiform bacill. When either alone were injected, no lesions were produced. The invasion of pyogenic

cocci was believed to be a secondary phenomenon. Smith (1932), however, believes that other anaerobic organisms, especially streptococci and vibrios, are also concerned in the infection. He was unable to produce lesions with cultures of any of the organisms separately, but only with mixtures of all of them. He has also shown the identity of the organisms from oral and from pulmonary lesions. By intratracheal injection into rabbits he produced typical lung abscess or gangrene both with sputum from human cases and with membrane from cases of Vincent's angina.

Laboratory diagnosis depends upon the demonstration of the spirochaetes and fusiform bacilli in stained smears or by dark-field illumination in perfectly fresh material. If possible the membrane should be removed and preparations made from the depths of the ulcer. The fact that they are present in small numbers in the mouths of many normal individuals must be remembered in interpreting the findings, and other pathological conditions to which they may be secondary should be excluded. Sputum must be examined when fresh, since the spirochaetes may be autolyzed within an hour or two. The peculiar sickening, slightly sweetish odor to the breath in infections of the mouth and lungs may suggest the etiology. There is no increase in the granular leukocytes in the blood, and there is occasionally a marked lymphocytosis.

Treatment.—These organisms, like most spirochaetes, are susceptible to arsenic and bismuth preparations, and when local measures, such as oxidizing agents, do not suffice to clear up the condition, arsenic and bismuth locally and by injection are of great value. Arsenic should be given intravenously in pulmonary cases as soon as the diagnosis is made.

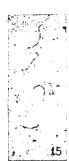
## Infectious Jaundice (Weil's Disease)

Leptospira icterohaemorrhagiae.—This organism is the type species of the genus Leptospira, first described by Noguchi. These organisms are not so distinctly blood parasites as are those of the relapsing fevers, but are intermediate between them and the true tissue spirochaetes. L. icterohaemorrhagiae is the cause of infectious jaundice (Weil's disease). The organism was first described by a group of investigators in Japan, where the disease is prevalent and severe. During the World War the organism was found in epidemics of the disease among the soldiers, and Noguchi showed that these European strains were immunologically identical with those from Japan.

The clinical picture of Weil's disease is not unlike that of yellow fever, in spite of the difference in the etiological agent and in the transmission of the two diseases. The onset of symptoms is abrupt, with rigors, fever, headache, vomiting, and muscle pains. The conjunctivae are hyperaemic. Albuminuria is present. After from 3 to 6 days jaundice appears with enlargement of the liver and spleen, and a tendency to haemor-

rhages into the skin or mucous membranes. In the pre-icteric period the *Leptospira* are practically always present in the blood, but they disappear rapidly in the icteric stage. At this time antibodies make their appearance in the blood. The organisms are very numerous in the liver and in the kidneys; and in the latter organ they may persist for weeks after convalescence, during which time they are excreted in the urine.

Transmission.—The infection occurs naturally in a high percentage of



rats in various parts of the world, even in regions in which human infection is rare. The rat apparently remains healthy, but the organisms are demonstrable in the kidneys. and are excreted in the urine. Field mice have been found to be infected, and the organisms can be kept for long periods in living mice, but the natural reservoir of the organisms is the rat. Man is secondarily infected by contact with soil or food which is contaminated by the urine of affected rats. It has been demonstrated that the Leptospira can penetrate the skin even when there are no apparent abrasions, and it is believed by many that this is the usual atrium of infection. It is a recognized fact that the disease is particularly prevalent in wet areas, and it has been suggested that a water-sodden skin facilitates penetration by the Leptospira. They have been shown to survive in moist soil for as long as 3 months. Infection may also occur through the digestive tract, however, and Inada believes that this is the usual way.

FIG. 32.-Four specimens of Leptospira icterohaemorrha-They appear blunt curved and without indicaanv tion of the minute mentary spiwhich are the characteristic feature of thisge-(After nus. Noguchi Journal of ExperimentalMedicine.)

Morphology.—The Leptospira icterohaemorrhagiae vary considerably in size—6-14µ (even 4 to 20µ) by 0.25µ. Noguchi showed that the thread-like body of the organism is made up of a large number of minute, tightly coiled elementary spirals. There are often several coarse, irregular undulations of the body, and one or both of the ends frequently show a characteristic hook. There are no terminal flagella. Propulsion seems to occur by a rotary 1 otion on the long axis and by undulations. When only one end is hooked, progression is in the direction of the straight end. It is best seen by dark-field illumination, but can be demonstrated with Giemsa's stain or by one of the silver impregnation methods.

Cullivation.—These organisms can be cultivated on Noguchi's leptospira media. They prefer a partial oxygen tension, and usually grow in a narrow zone just below the surface. The optimum temperature for growth is from 25 to 30°C., unlike most pathogens.

The Leptospira are able to pass through the ordinary types of Berkefeld filters (N, V, W). Inada has obtained filtrates in which no spirochaetal forms were demonstrable, but which were infective for guinea pigs. He believes, therefore, that there may be a viable, granular form.

Animal inoculation.—Young guinea pigs are particularly susceptible to the disease, and die within 10 or 12 days with characteristic pathological lesions. The tissues are bile-stained, and the pleural and peritoneal surfaces are dotted with haemorrhages. The liver is enlarged and is teeming with organisms which are easily visible in a dark-field preparation. The virulence of this Leptospira is easily lost, but can be restored by passages through guinea pigs.

Immunity.—By the end of the first week of the disease antibodies become demonstrable, and with their development there is a decrease in the number of organisms in the body and in their infectivity. Convalescent sera will protect guinea pigs from an otherwise fatal dose of the Leptospira. The immune serum contains lysins which cause a breaking



Fig. 33.—A Leptospira viewed under the dark-field microscope, showing its minute elementary spirals. (After Noguchi in Journal of Experimental Medicine.)

Fig. 34.—Showing Leptospira. (After Noguchi, in Journal of Experimental Medicine.)

Fig. 35.—A group of Leptospira icterohaemorrhagiae from a culture, stained by Pontana's method. (After Noguchi in Journal of Experimental Medicine.)

up of the organisms in the animal body (Pfeiffer's phenomenon), and also in vitro if fresh serum is used.

The adhesion (or thrombocytobarin) phenomenon is said to differentiate sharply the various species of Leptospira. It depends upon the fact that the organisms are altered by their homologous antiserum in such a way that small particles in the mixture adhere to their surface. The nature of the particles is immaterial—platelets, leukocytes, living or dead bacteria, or inorganic substances. The serum must be fresh, or reactivated by the addition of a small amount of complement. The phenomenon is a manifestation of a specific antigen-antibody reaction, and depends upon some physico-chemical change in the antigen. The technique is simple. Fresh, undiluted antiserum is mixed with an equal quantity of a suspension of organisms and E. coli. After standing for 20 minutes a dark-field preparation is examined. If the serum is homologous, the colon bacilli will be crowded around the surface of the spirochaetes. Control preparations are necessary. This phenomenon hus been obtained with trypanosomes as well as with spirochaetes.

Laboratory diagnosis.—The L. icterohaemorrhagiae can be found in the blood only during the first few days of the disease. They may be seen in Giemsa-stained thick blood films or in fresh preparations by dark-field

illumination. They are rarely numerous, however, and it is usually necessary to inoculate a guinea pig (3 to 5 cc. of blood intraperitoneally) in order to demonstrate them. After the animal dies they can always be found in large numbers in an emulsion of the liver.

After the first week of the disease they should be searched for in the urine. The centrifugalized sediment can be examined directly, and by guinea pig inoculation. Inada has found that practically all cases of the disease show organisms in the urine by the 20th day. If cultures of the organism are available the presence of antibodies in the serum may be tested by protection tests or by the Pfeiffer reaction.

Vaccination and serum treatment.—In Japan prophylactic vaccination with killed organisms has been tried with apparently good results. Immune sera have been obtained from horses and used in treatment. The results are said to be favorable if given early in the disease. After the characteristic symptoms have appeared, however, serum treatment seems to have little or no effect.

In some cases of a disease of dogs known as "yellows" an organism has been found which is apparently identical with the *Leptospira icterohaemorrhagiae*.

Leptospira hebdomadis.—This organism is the cause of seven-day fever, a disease which occurs in parts of Japan and resembles a mild Weil's disease. The organism cannot be distinguished morphologically from that of Weil's disease, but Ido, Ito, and Wani were able to differentiate it by cross protection tests and by the Pfeiffer phenomenon. They found that the organism was carried by field mice and transmitted to man by contamination of the soil with the urine of the infected animals. It is only feebly pathogenic for animals.

Leptospira autumnalis.—Similar organisms have been found in cases of the disease known as "autumn fever" in Japan. Koshina, Shiozawa and Kitayama have differentiated 2 species by their serological reactions. One was found to be identical with L. hebdomadis. The other, L. autumnalis, was more virulent and resembled the L. ictero-haemorrhagiae, but did not correspond to either serologically.

## Rat Bite Fever

Leptospira morsus-muris (Spirillum minus).—Futaki and his colleagues (1916) first demonstrated that this organism is the cause of rat bite fever. A week or two after the bite of an infected rat, fever develops which is relapsing in type and may continue for long periods. The site of the bite becomes inflamed, and the regional lymph nodes enlarge. After a few days a maculo-papular rash appears which tends to recur with succeeding paroxysms of fever. Organisms are present in the local lesion and the adjacent lymph glands, and occasionally in the blood in the acute stage. This infection is amenable to arsenic (arsphenamine) therapy. The disease is prevalent in Japan, and occasional cases have been reported from many countries including the United States.

Transmission.—In an infected rat the organisms are found in the connective tissue, particularly around the lips, tongue, and nose. They have not been found in the saliva, and the transfer of infection by the bite appears to depend upon the existence of some break in the mucous membrane around the mouth. Mooser, working with experimental animals, has noted the frequent occurrence of infections in the eye and was able to find organisms in the conjunctival secretion. He suggests that this may be the source of the infection transmitted by the bite.

Morphology.—This organism is very variable in size. Most of them range from 2 to  $5\mu$  by about  $0.2\mu$ , but much longer forms are occasionally seen. The coils vary in number depending upon the length of the organism, and are uniformly spaced so that their crests are approximately  $1\mu$  apart. The body is relatively rigid, and one or more flagella are present at each pole. The organisms can be stained readily with the usual aniline dyes, or by one of the Romanowsky blood stains. Silver impregnation methods are used to demonstrate them in the tissues. The motility observed in dark-field preparations is unlike that of other Leptospira, and resembles the rapid, darting movements of the vibrios. The organism remains rigid and is apparently propelled by the flagella.

Since this type of locomotion is unlike that of other spirochaetes, some bacteriologists consider that this organism should be placed in the genus *Spirillum* and refer to it as *Spirillum morsus-muris*, or *Spirillum minus*.

Culture.—Futaki believed that he obtained growth of the organism in special media, but others have not been successful.

Immunity.—Ido, Wani and Okuda demonstrated the presence of bacteriolytic antibodies in the serum of convalescents.

Laboratory diagnosis depends upon the demonstration of the organism at the site of the bite or in material aspirated from a regional lymph gland. Occasionally they may be found in the blood in early cases. When numerous they may be seen in smears from the material, but they are more readily demonstrated by animal inoculation. Guinea pigs are susceptible and usually succumb to the infection. The organisms can be demonstrated in the blood without difficulty. Rats and mice are also susceptible, but the blood invasion is transient, and they rarely, if ever, die from the infection.

## TISSUE SPIROCHAETES

The spirochaetes of syphilis and yaws may be distinguished from other genera of spirochaetes by the rigidity of their spirals and by their biological affinity for the tissues rather than the blood. Although both the *Treponema pallidum* and the *Treponema pertenue* (if this be regarded as a distinct species) have been demonstrated in the blood by infectivity tests, their presence here is transient; and it is in the tissues, particularly the lymph nodes, that they are found in demonstrable numbers over long periods.

## Syphilis

Treponema pallidum.-Schaudinn and Hoffmann, in 1905, first described this organism which they demonstrated in the primary lesion and in the swollen lymphatic glands in syphilis.

Morphology.—T. pallidum is a slender, delicate spirochaete ranging from 3µ to 15µ (average 10µ) in length by about 0.3µ in width, with tapering ends. There are from 6 to 12 relatively rigid, tightly coiled, small spirals regularly spaced about 14 apart. Many individuals show a bend in their long axis. The organism is best seen in dark-field preparations, in which they appear as silvery white coils which remain sharp and white

> when the focus is changed. While in motion the spirals appear continuous, but when at rest they often present the appearance of a series of silvery dots and dashes. Progression is due primarily to a rotary movement with slight undulations, with some expansion and contraction of the coils. Motility is less active than that of most of the spirochaetes. This organism cannot be stained with the ordinary aniline dyes, but is well demonstrated by





Fig. 36. Fig. 36.—Treponema pallidum.

Fig. 37. Fig. 38. Stained smears. (After Noguchi in Journal of Experimental Medicine.) Fig. 37.—Treponema pallidum. Darkfield. (After Noguchi in Journal of Experimental Medicine.)

Fig. 38.—Treponema pallidum. Fontana's stain. (After Noguchi in Journal of Experimental Medicinc.)

prolonged immersion in Giemsa's stain, or better by Fontana's silver impregnation method. Levaditi's method is recommended for sections.

The organism is not filtrable. It has been claimed on morphological grounds that a granular or filamentous "prespirochaete" phase occurs, but evidence of this has not been substantiated.

Cultivation.—Noguchi first reported the cultivation of the T. pallidum on a special medium incubated anaerobically. (See section on media.) Isolation was accomplished by inoculating a deep tube of the medium with a stab. After the spirochaetes had spread through the medium transplants were made from the peripheral part of the medium. From such cultures he produced more or less typical lesions in animals. A number of investigators, however, have failed to confirm these results, and have found that when spirochaetes were obtained in cultures they were entirely avirulent for animals. Zinsser concludes, therefore, that the culture strains either belong to a different species or are dissociated forms of T.

Pathogenicity for animals.—Monkeys are susceptible to the disease, and typical primary and secondary lesions may develop. They may be inoculated subcutaneously, or by rubbing material on a scarified area on the eyebrows or genitals. In rabbits a syphilitic orchitis may be produced by intratesticular injection of syphilitic tissue or scrapings from a chancre. Occasionally a chancre-like skin lesion appears in from 3 to 6 weeks at the site of the puncture. Inoculation into the anterior chamber of the eye causes a keratitis and iritis, and localized lesions elsewhere can be produced occasionally. Living spirochaetes can be found in large numbers in the lymph glands over a long period of time. In working with rabbits, it is important to recognize that they are subject to a natural venereal disease, the etiological agent of which (T. cuniculi) may be morphologically indistinguishable from the T. pallidum.

Clinically the disease is divided into three more or less clear cut stages, the manifestations of which depend in part upon the distribution of the organism, and the reaction of the tissues to its presence. One or more of these phases may be absent, and in some cases there may be no manifestations of the disease for many years. In the so-called "latent" cases the diagnosis depends solely upon serological reactions.

After an incubation period of from 3 to 6 weeks a *primary sore* or chancre appears at the site of inoculation, and the regional lymph nodes enlarge. Spirochaetes are present in these areas in large numbers, and from here they become widely distributed throughout the body. They occur in the blood stream, although not in sufficient numbers to be demonstrated directly. It is believed that the organism gains entrance to the nervous system at this time in those cases which later develop neurological manifestations.

Characteristic lesions on the skin and mucous membranes mark the onset of the secondary stage of the disease. Spirochaetes are present, and can be demonstrated, in these areas. By this time characteristic serological changes have developed in a great majority (95%) of the cases, and are relied upon to confirm the clinical diagnosis.

After a variable length of time the secondary manifestations disappear. The spirochaetes become much less numerous in the body, but generally persist in localized areas with or without the development of symptoms. Such a nidus may occur in almost any tissue in the body, and may produce gross pathological lesions within a short time, or only after many years. As a result of these peculiarities in the infectious process, the manifestations of tertiary syphilis are of many different types, and occur almost anywhere in the body. The symptom complex of many unrelated diseases may be simulated by a syphilitic infection, and can be differentiated only by serological tests. While spirochaetes may be present in these tertiary lesions for many years they are usually sparse and are not easily demonstrated even in tissue sections. The organisms are frequently harbored in the lymph nodes which have been shown to be infective for rabbits, even in latent cases many years after the initial infection. Lansford and Day (1934) have tested the infectivity of the nodes in a series of Wassermann-positive cases in different stages of the disease. In the primary cases glandular transfer was positive in all; in cases receiving treatment at the time all were negative. In late cases, however, with or without previous treatment, transfer was successful in only about 35%. These results indicate that failure to obtain infection in rabbits by inoculation from human lymph glands is not a reliable criterion of cure, as has been suggested.

It has been claimed, chiefly upon clinical observations, that certain so-called *neurotropic strains* of *T. pallidum* possess a particular affinity for the central nervous system. There is, however, no conclusive proof of this contention, and the factors determining the type and location of the tertiary manifestations are not well understood.

Syphilis may be congenital, in which case the spirochaetes may occur in great numbers in the internal organs, particularly the liver. They have been demonstrated in the blood, and are present in the placental lesions. Blood from the cord may show a positive Wassermann reaction. Adequate treatment of the mother before or during pregnancy will prevent the disease in the child, and if the foetus is already infected may combat the disease in utero.

Immunity.—The problem of immunity in syphilis is but little understood. At no stage of the disease are the ordinary antibodies such as agglutinins, or bacteriolysins, demonstrable in the serum according to most observers, and it does not possess any protective power for animals. In the tertiary stage allergic skin reactions may be obtained with spirochaete extracts (luetin) and to other protein extracts, but they are not specific, and the luetin reaction is no longer utilized as a diagnostic procedure.

Serological Reactions.—Serological changes develop, however, which are of the highest diagnosic importance, although their nature is not understood. Within from 2 to 4 weeks after the development of the primary lesion the serum acquires the property of reacting specifically with an emulsion of certain lipoidal substances from normal tissues. This reaction may be manifested by the development of flocculation in a mixture of serum and lipoid emulsion, and by the demonstration that when complement is added to such a mixture fixation occurs. This altered reactivity of the blood is present in practically all cases in the secondary stage of the disease and in untreated congenital cases, but may be absent in a minority of the tertiary and latent cases. When the central nervous system is involved the reaction is demonstrable in the spinal fluid although the blood may be entirely negative.

Details of the Kahn flocculation test and the Wassermann complement fixation test and their interpretation are given in chapter XII.

The American Serological Conference has recently compared the results of the different tests on the basis of their sensitivity and specificity, and have concluded that flocculation and complement fixation tests are equally reliable when properly performed. Although the test is practically specific, there are a few other conditions in which positive reactions may be obtained. In yaws the percentage of positive results seems to be as high or higher than in syphilis; and "false positives" are obtained frequently in leprosy, and occasionally in malaria.

This serological change is associated in some way with the presence of spirochaetes in the body and is not an index of immunity. The actual defense mechanism depends primarily upon some obscure alteration in the capacity of the tissue cells to react to the virus rather than upon humoral antibodies. The immunity which develops is rarely (if ever) effective in ridding the body of organisms but is usually sufficient to prevent

superinfection. Although it seems to persist for some time after the disease is apparently cured, it is not life-long since a second attack of the disease may occur.

The problem is complicated by our inability to determine when the disease is actually cured. Various criteria of cure have been suggested, but none is entirely satisfactory. Syphilitic individuals are refractory to a superinfection, and the development of a re-infection has been regarded as a proof of cure. It is difficult, however, to prove that re-infection has not taken place, since many cases of infection occur without a recognized local lesion. Furthermore, it is possible that sufficient immunity to prevent re-infection may persist for some time after a patient is cured. The demonstration of lymph node sterility by animal inoculation is so inconstant in human beings that it seems to be of little value as an indication of cure (Moore, 1935).

Laboratory Diagnosis.—In the primary stage of the disease the diagnosis is established by demonstrating the *Treponema pallidum* in smears from the chancre. To obtain material for examination cleanse the lesion with alcohol and allow it to dry. Rub the margin of the ulcer with gauze, or scrape it gently with a scalpel so that serum may be obtained from the corium. If bleeding occurs the surface should be wiped gently until the exudate is clear. Gentle squeezing is permissible. Material may also be obtained by washing the lesion, and applying a Bier cup or a test tube which has been warmed so that upon cooling a partial vacuum develops.

This exudate should be examined fresh with dark-field illumination. The T. pallidum is easily seen, but must be distinguished from other non-pathogenic spirochaetes (particularly T. refringens) by its tight corkscrew spirals and relatively sluggish motility.

When the material cannot be examined immediately, thin smears can be made and stained. Fontana's method is excellent. The Warthin-Starry method is good. The India ink method of Burri is simple and can be recommended. The material is mixed with a small drop of drawing ink, and thin smears are made as for blood. When dry they are examined with the oil-immersion lens, and the spirochaetes stand out as white spirals against a dark background.

As the *primary* lesion begins to heal the organisms become less numerous and may be difficult to demonstrate. At this stage, however, they are present in large numbers in the regional buboes, and can be found in the fluid obtained by gland puncture. Although spirochaetes may occur in the blood in the primary stage of the disease, their demonstration here is not a practicable diagnostic procedure. The fact is of great importance, however, in selecting a donor for transfusion, since syphilis has been transmitted in this way in a number of instances. At this stage serological tests are usually negative, and the condition may escape notice unless the donor is carefully questioned and examined.

In the secondary stage the spirochaetes occur in the serous exudate obtained from mucous patches or from skin lesions. The spirochaetes

#### STUDY AND IDENTIFICATION OF SPIROCHAETES

which occur in normal mouths  $(T.\ microdentium)$ , however, can not be distinguished morphologically from  $T.\ pallidum$ , but at this time the serological tests are practically always positive, and the demonstration of the organisms is not necessary for diagnosis. In the tertiary lesions they are usually sparse, although they have been found in sections from a number of different tissues.

After the primary stage the complement fixation and flocculation reactions in the serum are relied upon for diagnosis.

When central nervous system involvement is suspected the spinal fluid must be examined, even though the blood may be negative. In addition to the serological tests the following examinations are particularly important: (1) cell count; (2) globulin and total protein estimation; (3) quantitative estimation of glucose; (4) colloidal gold or mastic tests. These procedures are described in Chapter XXXII. No case of syphilis should be considered cured until the spinal fluid as well as the blood has been examined and found to be normal.

Certain procedures in the *treatment* of syphilis requiring special technical knowledge may be mentioned briefly, although the subject as a whole is beyond the scope of this book.

The Swift-Ellis treatment has proved of value in some cases of cerebrospinal syphilis, particularly in cases of tabes dorsalis associated with lightning pains and gastric crises. The technique is as follows. One hour after an intravenous injection of arsphenamine withdraw about 50 cc. of blood, and allow it to clot in the ice-box over night. Separate the clot and centrifugalize until the serum is perfectly clear. Pipette off 12 cc. of serum and add 18 cc. of sterile physiological saline. Heat the mixture in a water bath at 56°C. for an hour. A lumbar puncture is performed and approximately 30 cc. of cerebrospinal fluid is allowed to drain out. The serum mixture is immediately introduced, very slowly, by gravity. Strict asepsis must be observed throughout.

Fever therapy is used in conjunction with the usual chemotherapy in late neuro-syphilis, and in refractory cases. Remarkable clinical results have been obtained, particularly in general paresis. The mode of action of the fever is obscure, but it is believed to stimulate in some way the defensive mechanism of the body.

Various methods of producing a pyrexia are used. Of these the original and most widely used is the production of an attack of malaria by inoculation with blood containing parasites. The benign tertian type is most commonly used, although the quartan and even the aestivo-autumnal parasites have been used (see Chapter XVIII). From to 5 cc. of blood (citrated to prevent clotting) are obtained from an active case of malaria (naturally or artificially infected) and injected intravenously or intramuscularly. So far as is known, there is no danger in injecting blood from one syphilitic individual to another, particularly when the donor has received recent and thorough chemotherapy, although a non-syphilitic donor is naturally preferred if available. The malaria is produced in some clinics by inoculation from the bites of infected mosquitoes. This method has some advantages, but it involves a great deal of time and labor to breed, care for, and infect the insects.

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Artificial infections with the spirochaetes of relapsing fever or rat-bite fever have been used in place of malaria, but these diseases are not always so easy to control therapeutically as is tertian malaria.

Pyrexia may also be produced by the intravenous injection of a foreign protein such as typhoid vaccine. The initial dose should be about 50 million organisms, and subsequent quantities must be determined by the response of each individual patient. Schnitker (1934) advocates a filtrate containing only the H (flagellar) antigen, and claims that the febrile response is more constant and prolonged, and the constitutional symptoms are milder.

Various physiotherapeutic procedures such as diathermy (or short-wave diathermy), infra-red radiation, hot air or hot baths, are also used to induce fever.

All types of fever treatment must be followed by appropriate chemotherapy, preferably arsphenamine, to obtain any lasting improvement. All of the above methods seem to be effective in some cases, and it is a matter of opinion which is to be preferred.

## Yaws (Framboesia)

Etiology.—Yaws is an infectious, highly contagious, non-venereal disease, limited to the tropics and caused by an organism which is morphologically indistinguishable from, if not identical with T. pallidum of syphilis. Blacklock refers to framboesia as "tropical syphilis," and lists the term T. pertenue as a synonym of T. pallidum. The organisms are found in large numbers in the serous discharge from yaws lesions and in the lymph glands. They also occur in the blood, as shown by inoculation experiments. Several observers have reported cultivation of the organisms by Noguchi's method, although some recent attempts (Johnel, 1934) have been unsuccessful. Turner (1936), however, obtained cultures of yaws spirochaetes, preserved frozen at about  $-78^{\circ}\mathrm{C}$ ., with which he was able to infect rabbits.

In practically all text books, yaws is described as a disease separate from syphilis. Condylomatous lesions of the perineal region are common in yaws, and the disease may be transmitted venereally. Brickell (1737) noted that in North Carolina yaws was very prevalent in the African slaves of that colony. Yaws was also extremely common in the West Indian slaves brought from Africa. Jonathan Hutchinson noted that when the white man contracted yaws in the tropical colonies, he came home with syphilis. Just as we have yaws in the natives of the rural districts of Haiti, and syphilis in the natives of the adjacent towns, it would seem that the yaws of the African slaves of the United States is now continuing as syphilis. Of course syphilis was also introduced by the European colonists, and some of the slaves may have contracted syphilis from the white man, but this infection may have been minor as compared with yaws. We consider that the evidence for the unity of syphilis and yaws as presented by Admiral Butler is very convincing.

Animal Inoculation.—Monkeys and rabbits can be infected by inoculation with discharges from yaws lesions. Schöbl (1928) obtained primary lesions in monkeys by inoculation of the eye brows or (more readily) of the scrotum. By a re-inoculation

while the primary lesion was healing (superinfection) he obtained a generalized secondary eruption closely resembling yaws in man. In rabbits most observers have described dry lesions following skin inoculation with yaws, whereas with syphilis they resemble chances. Turner and others reported that the epididymo-orchitis in rabbits following inoculation with yaws is much less marked than with syphilis.

Epidemiology.—Yaws is limited to the tropics, and is especially prevalent in the West Indies, Africa, south-eastern Asia, the East Indies and the Pacific Islands. It is almost exclusively confined to colored races. It is largely limited to rural districts in which syphilis is rare, whereas in the towns vaws is uncommon and syphilis (venereally acquired) is prevalent. Infection is usually acquired innocently in childhood. The spirochaetes enter the body through some cut or abrasion of the skin. either by direct contact with discharges from the lesions or indirectly, particularly through the agency of flies. Krumm and Turner (1936) showed that rabbits could be infected with yaws by scarifying the skin and exposing them to Hippelates pallipes which had fed on infective discharges (see p. 540). These flies were observed in swarms feeding on the discharges from yaws lesions. In Jamaica the curve of incidence of new cases parallels the rainfall and (consequently) the prevalence of *Hippelates*. The spirochaetes apparently do not penetrate the unbroken skin. The rarity of the disease in white children may be explained in part by the fact that they are kept clothed and clean.

Clinical Picture.—A primary, secondary, and tertiary stage of yaws can be distinguished. The primary lesion (mother yaw) develops three and a half to four weeks after experimental infection (Sellards) and one to two months after natural infection. It appears as a small papule which attains a diameter of about 1 cm. or more and becomes covered with a crust of dried, serous exudate. If the crust is removed it reveals a fungoid, yellowish or reddish tubercle which exudes a serum containing spirochaetes (framboesia). Irregular fever, malaise, joint pains, and nocturnal headaches precede the appearance of the primary lesion which is usually located on the legs. This may regress spontaneously after a few weeks or it may persist for many months. There is a regional, and later a general, glandular enlargement. From 2 to 3 weeks after the primary lesion appears, the Wassermann reaction becomes positive.

Six to twelve weeks after the primary lesion a generalized sccondary cruption develops, first as small, dry, scaly patches in which small papules soon appear. These enlarge and resemble the primary lesion. There is a recurrence of fever and constitutional disturbances. The lesions itch but are not sensitive or painful except on the palms and soles. The disease is called "crab yaws" when the lesions occur on the soles because of the resulting peculiar, awkward gait. There may be successive crops over a period of several months or years. The individual lesions usually regress after several weeks, leaving a thickened, subsequently pigmented scar.

In about 8% to 10% of the cases the lesions persist as chronic ulcers (tertiary stage) which may invade the neighboring tissues extensively. Osteitis and periostitis are

common and may involve the fingers as in syphilis, causing marked deformity. Other late lesions include:

Gangosa.—The term gangosa is applied to a chronic ulceration resembling that sometimes seen in syphilis, which starts in the palate and gradually destroys the soft parts and bones of the roof of the mouth and the nose (but spares the upper lip), causing frightful mutilation.

Juxta-articular nodules of fibrous tissue, varying in size from that of a pea to a small orange, often occur on the extensor surface of the limbs, especially about the joints.

Goundon, probably also a tertiary manifestation of yaws, is characterized by a progressive, symmetrical enlargement of the nasal processes of the superior maxillae, which eventually form large projecting tumor masses.

Immunity.—An individual who passes through the secondary stage of yaws acquires a substantial degree of immunity to superinfection, but this seems to develop more slowly than in syphilis. There is convincing evidence that an individual who has acquired an immunity to yaws is also immune to syphilis, and vice versa. In Guam naval medical officers repeatedly have noted the striking immunity to syphilis of the natives, almost all of whom have had yaws in childhood, and Daniels observed the same fact in Fiji. Hudson (1936) described in the desert Arabs of Syria a form of syphilis (Bejel) largely acquired innocently in childhood, which closely resembles yaws except for the absence of a primary yaw, but found no venereal syphilis among them. The latter disease, however, was prevalent among the Arabs in the towns.

Attempts by Johnel and Lange to inoculate paretics with two strains of yaws spirochaetes were unsuccessful, although a control case of multiple sclerosis became infected.

Relation to syphilis.—The exact relationship of yaws to syphilis is still in controversy. Those who maintain that the diseases are distinct point out differences in clinical manifestations and in the lesions in experimental animals. They have emphasized: (1) The absence of mucous patches in yaws. Carter, however, found no mucous patches in 231 American negroes with syphilis, although he found 21 cases among an equal number of white patients. (2) The rarity of tabes and paresis in yaws. These conditions are also rare in syphilis among primitive peoples in the tropics. (3) The reported absence of typical aortitis. Recent observations, however, show that aortitis is not uncommon, and Carl Weller (1936) from a study of the aortas of 169 cases in Haiti, a majority of whom had had yaws, concluded that yaws and syphilis produce identical aortic lesions. Hazen (1936) has emphasized the frequency of annular lesions and condylomata in the syphilis of the American negro. This annular or circinate type of lesion is frequently noted in yaws (ringworm yaws).

The morphological identity of the organisms, the common serological reactions, the cross immunity, the response to the same therapy, as well as the general similarity of the experimental lesions and the clinical

manifestations indicate that the diseases are, at least, very closely related. For years in numerous articles and in his monograph on Syphilis (1936), C. S. Butler has insisted that yaws is syphilis, modified by race, climatic influences, immunity, extragenital infection in childhood, and absence of specific treatment.

Laboratory Diagnosis.—In the primary and secondary stages the organisms may be found in large numbers in serum expressed from the lesions or in fluid aspirated from enlarged lymph glands, preferably examining fresh preparations with dark-field illumination. After the third or fourth week the Wassermann reaction becomes positive in over 80% of the active cases. The Kahn reaction is equally reliable.

Treatment.—The drugs commonly used in the treatment of syphilis are equally effective in yaws. The direct mortality is very small.

## CHAPTER VIII

## DISEASES DUE TO RICKETTSIA AND BARTONELLA

#### RICKETTSIA INFECTIONS

In ever increasing number, diseases caused by Rickettsia infections have been reported from all parts of the world since Ricketts, in 1908, described very small chromatin-staining lanceolate bacteria in human cases of spotted fever of the Rocky Mountains and in the ticks which transmit it. Subsequently, in 1910, Ricketts noted similar organisms in lice infected with Mexican typhus.

There are five main groups of human rickettsial diseases, all transmitted by arthropods.

- r. Classical Typhus of Prison and Famine Epidemics.—This is transmitted from man to man by the body louse. To this group belong the tabardillo or Mexican typhus of the highlands of Mexico, the typhus epidemics of Chile and Peru, Northern China and Indo-China. The European focus is in Russia and the Balkan States. This group is often referred to as epidemic typhus or louse-borne typhus. Whether cases of Brill's disease in New York are relapse cases of European typhus, as thought by Zinsser, or are identical with endemic typhus in etiology is not certain at present.
- 2. Endemic Typhus.—This is a mild type of typhus which was studied intensively by Maxcy in the Southern United States and later recognized as wide-spread over the world. It is kept alive in nature in rats, and is transmitted from rat to rat, and from rat to man by rat fleas. It is similar to plague in its epidemiology. The rat louse as well as the flea transmits the disease from rat to rat, but the rat louse does not bite man. The infection is introduced by scratching or rubbing the faeces of the flea into the skin, but not directly by the bite. The Rickettsiae multiply greatly in the flea, so that after about a month one flea may contain enough to infect a million guinea pigs. The mortality of this type is under 5%, whereas that of European typhus is more than 25%. Endemic typhus has been reported from seaports all over the world—North, Central and South America, the Mediterranean basin, South Africa, Manchuria and Cochin-China. It is often referred to as murine

typhus on account of its association with rats. It is a disease of summer and fall (when rat fleas are most numerous), whereas epidemic typhus is a disease of winter (when the body louse is prevalent).

Rickettsial infections of the louse, including *Polyplax spinulosus*, the rat louse, which is an important transmitting agent from rat to rat, are regularly fatal to the louse within two weeks, whereas the rat flea, *Xenopsylla cheopis*, apparently is not harmed by the infection.

3. Spotted Fever of the Rocky Mountains.—This disease, first described by E. E. Maxey, in 1899, was for many years supposed to be confined to the mountainous regions of Idaho and Montana, with occasional cases in Wyoming, Utah, Nevada, and California, but in 1930 the Public Health Service established the fact of its existence in the Alleghany region of the U. S., and it has now been reported from almost all of the states. It is definitely established that the exanthematic typhus of São Paulo, Brazil, is the same disease, and it is very probable that boutonneuse fever, of countries bordering the Mediterranean, is closely related. There is also a close relationship to the tick-bite fever of South Africa.

Transmission of the disease in the states of Western U. S. is by the tick *Dermacentor andersoni*, the common wood tick of that region. The virus is transmitted to the egg, so that the infection can be kept up in the tick without another reservoir of virus. However, numerous animals of this area are susceptible, and undoubtedly play a part in the transmission of the disease to man, although none have been found naturally infected. Unlike epidemic typhus, man has no rôle in the transmission of this disease. In the Eastern states *D. variabilis* is the natural vector. It is the common dog tick of that region. Only adult ticks infect grown persons, but nymphs may feed on children.

In South African tick-bite fever, Rhipicephalus appendiculatus, Amblyomma hebraeum, and Boophilus decoloratus are vectors, and the virus reservoir is thought to be in the small rodents of the veldt. The transmitting tick of boutonneuse fever is a tick of domesticated animals, Rhipicephalus sanguineus. Infections may occur through the conjunctiva from contamination of the hands in picking ticks off man or dogs, as well as through the better known tick bite. The dog is thought to be the important reservoir of "boutonneuse" virus, although Brumpt has found this tick on 37 other hosts. Experimentally the dog does not show any symptoms, even when his blood conveys the infection.

- 4. Tsutsugamushi or Japanese River Fever.—This typhus-like fever is transmitted by a larval mite, *Trombicula akamushi*, which feeds on the field mice of the river banks. The mice serve as the reservoir of virus. Adult mites do not attack man. Mite-borne typhus has also been reported from Sumatra—"Scrub" typhus.
- 5. Trench Fever.—This influenza-like disease was first noted in 1915 in the allied troops serving at the front in the trenches. In the German forces the disease was known

as Wolhynian fever. Clinically, it was characterized by sudden onset, headache, muscular pains in the limbs and cyeballs, hyperaesthesia of shin bones, and a tendency to relapse. The disease is transmitted by the body louse, not from its bite but from contamination of an abrasion of the skin with louse faeces. The material resulting from crushing the louse in scratching the body is also infective. Scabies cases were especially liable to infection. According to Byam, lice may become infected when feeding on a convalescent more than a year after the original attack. Rocha-Lima, in Germany.

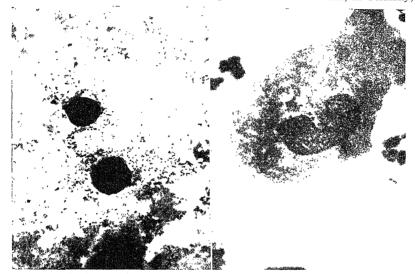


FIG. 39.—Rickettsiae from endemic typhus. Left: Smear of gut of flea (X. cheopis). Right: Scraping of guinea pig tunica showing intracellular and extracellular Rickettsiae. Giemsa stain. X 945. (Courtesy of Dr. R. E. Dyer, National Institute of Health.)

and Arkwright, Bacot, and Duncan, in England, rather definitely established the rickettsial nature of this disease and the name *Rickettsia quintana* has been given the virus.

The virus of rickettsial diseases.—Rickettsia prowazeki, the organism of epidemic typhus, is the type species of the genus. The organisms are diplococcoid, and show great variation in size and shape, from almost invisible particles to bodies approaching  $2\mu$  in length. Stained with Giemsa they take a purplish tint, and with Castaneda's stain they are light blue in color. These tints are unlike those of ordinary bacteria stained by these methods. They are not cultivable on ordinary media, but grow in the cells of tissue cultures, especially of Goodpasture's fertile egg medium.

It is possible that this genus was originally adapted to plants, later becoming parasites of mites feeding on plant juices, and subsequently infecting rodents on which the mites feed. From infected rodents, lice, ticks, and fleas became of importance in rickettsial epidemiology.

Although found in the blood and all organs of infected animals and man, they show a striking selectivity for cells of mesothelial origin, especially these cells of the tunica vaginalis, peritoneal cavity, and intima of small blood vessels.

Provisionally, besides R. quintana and R. prowazeki, we may accept R. mooseri as the virus of endemic typhus, R. orientalis as that of tsutsugamushi and R. rickettsi as that of tick-borne typhus-like diseases. However, the name proposed by Wolbach for the virus of spotted fever of the Rocky Mountains is often used—Dermacentroxenus rickettsi.

Symptomatology of Typhus-like Rickettsial Diseases.—It is less than a hundred years since Gerhard (1837) clearly differentiated typhus from typhoid fever, and there is evidence that cases of Rocky Mountain spotted fever, of Eastern U. S., were diagnosed as typhoid fever in competent clinics. With the recognition of endemic typhus these cases were confused with it, and only since 1930 has this disease been shown to be identical with spotted fever of the Rocky Mountains. Not only do tick-bite rickettsial diseases show great variation in virulence and symptomatology, but the same holds true even for classical typhus.

Almost all the rickettsial diseases show a rather rapid rise of temperature and a fastigium of approximately two weeks (in Rocky Mountain spotted fever, 3 weeks). The fever in endemic typhus has a rather abrupt decline, but with the tick and mite groups it falls by lysis. The eruptions show considerable variation, but have a tendency to become petechial. In typhus fever the eruption appears usually on the fifth to the seventh day, first on the lower ribs and upper abdomen, and spreads peripherally. The face is rarely involved. In Rocky Mountain spotted fever it appears on the third or fourth days on the ankles and wrists and spreads to the trunk and often to the face. In severe cases the spots become confluent and necroses may occur. The mortality in the western type may reach 75 to 80%; in the eastern type it is about 25%.

Cases of boutonneuse fever as well as tsutsugamushi show the local eschar at the bite site. The mental symptoms of epidemic typhus bear out the name of the disease—stuporous. In endemic typhus such manifestations are less marked or absent. In the tick and mite group excitement, insomnia, and hyperaesthesia are more characteristic.

Laboratory Diagnosis.—There have been numerous laboratory infections, chiefly in those who were working with infected lice, fleas, mites or ticks. These infections can be transmitted by injecting the blood of a patient into a man or laboratory animal, but infections from autopsies on human beings have been rare.

Practically, laboratory diagnosis of a rickettsial disease rests on (1) guinea pig inoculation and (2) the Weil-Felix agglutination.

I. The guinea pig.—This animal is regularly susceptible and has become a very important means of differentiating the rickettsias. The best method of inoculation is to inject I to 5 cc. of the patient's blood,

intraperitoneally, into a nearly grown male guinea pig—subcutaneous injection is unsatisfactory. In epidemic typhus, following an incubation period of 5 to 9 days, the guinea pig shows fever of 103.5 to 104.5°F. continuing for a few days. Either the blood of this guinea pig or an emulsion of his brain should infect another guinea pig. The brain of the guinea pig shows small proliferative nodules, with a perivascular infiltration. There are no gross scrotal or testicular lesions. On recovery the animal shows immunity to subsequent injections.

In endemic typhus, after a similar incubation period there is fever of 103.5 to 104.5°F. lasting 1 to 4 days, which appears with an erythema and swelling of the scrotal skin. The tunica vaginalis is adherent, and scrapings from it show Mooser bodies (*R. mooseri*). Characteristic brain lesions are present. The infection does not kill the guinea pig. There is a cross immunity with epidemic typhus.

In severe types of Rocky Mountain spotted fever the guinea pig also shows scrotal lesions, but these consist of thrombosis and necrotic oedema leading in virulent infections to sloughing or gangrene. There is an incubation period of 2 to 10 days, followed by fever lasting 5 to 15 days, with a mortality of 95%. In the milder eastern cases scrotal lesions do not occur. The mortality is 40 to 50%.

2. The Weil-Felix agglutination test.—The Proteus culture recommended by Weil and Felix, and universally used, is one isolated in 1916 from the urine of a typhus case, and known as Proteus X19. In a study of dissociation forms of this organism two forms were recognized, a motile, flagellated type (H) and a non-motile, unflagellated type (O). It is this latter type, or Proteus OX19, that is the standard culture. Other Proteus strains used for agglutination tests are OX2 and OXK. It is strange that the guinea-pig, infected with Rickettsia, fails to show serum agglutinins although protected from virulent doses of rickettsial material. Precipitins and bacteriolysins are also absent.

The test should be performed macroscopically. Serum dilutions of 1 to 25 and even 1 to 50 may fail to agglutinate (proagglutinoid zone) whereas higher dilutions (1 to 100 or 1 to 200) will show agglutination. Agglutinins are demonstrable by the end of the first week, and the titer rises during the course of the disease, often reaching 1 to 2000 at the termination of the fever, after which it steadily falls. A titer of 1 to 80 or less has no significance; 1 to 160 is suggestive; 1 to 320 or 1 to 640 is susually but not invariably diagnostic. Titers as high as 1 to 40,000 have been reported.

The Journal of the R.A.M.C. in an editorial (March, 1935) states that a classification of rickettsiae according to their vectors is unsatisfactory, and advocates a classification based on antigenic relations. The following table, based on the work of Felix, is given:

Epidemic typhus Tsutsugamushi Rocky Mountain

 $X_{19}$ 

X<sub>2</sub> XK

Bridges (1935) has noted that while the O variety of X2 is extremely stable this is not the case with these variants of X19 and XK, which tend to change to H variants. He points out the difficulties of maintaining standard cultures even in great national laboratories, and emphasizes the advantages of killed suspensions over the living organisms. Bridges recommends killing with alcohol, then removing the alcohol and resuspending in saline, then preserving with buffered formol saline, with the concentration of the formalin 0.25%.

Methods of immunization.—Spencer and Parker have prepared a vaccine to protect from Rocky Mountain spotted fever by grinding up in salt solution, containing 2% phenol, an emulsion of ticks recently fed upon infected guinea pigs. Blanc and his colleagues have used material obtained from the tunica vaginalis and spleen of guinea pigs infected with a strain of endemic typhus of low virulence and treated with ox bile.

Weigle's method of preparing vaccine against typhus consists of grinding up the intestines of infected lice in phenol and salt solution. Zinsser has prepared a vaccine against typhus by using suspensions of rickettsiae procured from the peritoneal cavity of infected rats.

#### BARTONELLA DISEASES

Carrion, a Peruvian medical student, inoculated himself with the blood of a verruga lesion and died with Oroya fever. This seemed to confirm the identity of the two diseases, but for many years a controversy continued as to unity or duality. In 1926, Noguchi and Battistini succeeded in cultivating on leptospiral culture-media Bartonella bacilliformis from a typical case of Oroya fever. Rhesus monkeys inoculated above the eyebrows with this culture developed typical verruga lesions. About the same time, Mayer and Kikuth excised verruga lesions from a patient, and produced in monkeys both verruga papules and Oroya fever. They were not able to isolate the organism in culture. The unity of the two diseases is now generally accepted, and the name Carrion's disease would seem appropriate. Strong and his colleagues (1915) named the parasite B. bacilliformis. It was discovered in 1909 in the red cells of a patient with Orova fever by Barton. Townsend (1913) reported the transmission of the disease by Phlebotomus verrucarum, and this work has been confirmed by others. Noguchi (1926) reported the possibility of transmission of the organism from monkey to monkey by a tick (Dermacentor andersoni).

Classification.—There are many views as to the classification of Bartonella. David Weinman in his monograph (1935) notes various taxonomic views which would classify them as piroplasms, rickettsiae, anaplasms, chlamydozoa or bacteria. Brumpt (1936) notes their near relation to Grahamella. Weinman divides Bartonella organisms into three groups: (1) B. bacilliformis, which causes a progressive haemolytic anaemia and a telangiectatic tumor. Only man and the monkey are surely susceptible. Rodents cannot be infected. Chemotherapy does not seem to influence the parasite. (2) B. muris of splenectomized rats. There is no associated tumor—only anaemia. Transmitted from rat to rat by the rat flea or louse. Arsenical therapy very effective. (3) B. rochalimai of non-splenectomized mammals. There are also bartonellas of cold-blooded animals (lizard).

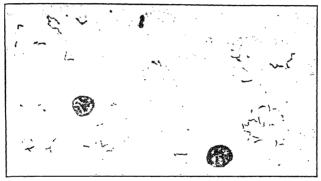


Fig. 40.—Bartonella bacilliformis in the peripheral blood of a patient suffering from Oroya fever. (After Strong, Tyzzer, Brues, Sellards and Gastiaburu from Brumpt.)

The organism.—With Giemsa stain B. bacilliformis shows as red bacillus-like bodies in the red cells, which at the height of the fever and anaemia may show multiple forms in most of the red cells. There also may be coccoid or dumb-bell forms and V shapes. In the endothelial cells of the verruga nodules the Giemsa stain shows red inclusion bodies which are made up of a mass of granules, which may be an evolutionary stage of the organism found in the red cells of the blood. Strong found that the bacillary form showed motility in dark-field preparations. The stained parasite suggests rickettsiae. Kikuth (1934) noted a relation between chronic malaria and Oroya fever and thought this might be connected with interference to defense forces of the damaged spleen. The influence of splenectomy on rats suggested this idea, but he was unable to confirm it in splenectomized monkeys.

Verruga.—This phase of *B. bacilliformis* infection is characterized by the appearance of very vascular tumors, at first showing newly formed blood vessels lying in an oedematous connective tissue. The lining endothelial cells may show more than a single layer. Around the blood vessels are angioblasts with frequent mitoses. The period of incubation in monkeys (experimental) is about two weeks, but the natural term in man is longer. The onset shows a moderate fever and severe joint pains. There are two types of lesions. (1) The miliary, in which numerous small wart-like tumors appear in

abundance on face and extensor surfaces of the extremities. Commencing as a bright red macule they become nodular or even pedunculated. This type may involve the mucous membrane. (2) The nodular type, where we have a very gradual development of nodules, which may become as large as a pigeon's egg. They chiefly affect the region of the joints.

Oroya Fever.—After an incubation period of three weeks, an irregular fever of remittent type sets in with an accompanying extreme anaemia, in which the red cells may drop to less than a million within a few days. As stated by Christophers, there is no other disease in which the red cell destruction is so rapid and extreme except blackwater fever. Erythroblasts are numerous. There is a leukocytosis with increase of metamyelocytes. The blood smear shows B. bacilliformis.

# CHAPTER IX

# FILTRABLE VIRUSES

The nature of the so-called filtrable viruses and their relationship to ordinary bacteria on the one hand and to inanimate substances, such as enzymes, on the other hand, are still controversial. This problem is complicated by the apparent impossibility of obtaining a pure virus, free from extraneous protein. A great deal has been learned in the last few years about the properties and activities of the various viruses, all of which is quite consistent with the assumption that they are living organisms comparable to bacteria. Although this opinion is held by a majority of the investigators, it cannot be considered as conclusively established. Arguments in support of the different views are summarized and discussed by Rivers (1932) in an excellent critical review on the nature of viruses.

## GENERAL PROPERTIES

Filtrability.—The viruses, on account of their minute size, are capable of passing through filters that are impervious to ordinary bacteria. (The technique of filtration and the various types of earthenware and porcelain filters are described on p. 821.) The passage of the virus through such a filter, however, depends not only upon its size, but also upon a number of physico-chemical factors in the filter and in the virus suspension. The chemical composition and viscosity of the medium, the duration of filtration, the degree of pressure used, the temperature at which filtration is carried out, influence the result. The relationship of the electrical charge of the virus particles (which is influenced in turn by the pH of the medium in which they are suspended) to that of the filter is a very important factor. Such suspensions, moreover, contain extraneous protein material upon which the virus may be adsorbed. Although filtration in this way affords a convenient method of obtaining the virus relatively pure and free from ordinary bacteria, it is impossible to draw precise conclusions as to its size from the porosity of these filters.

The ability to pass through such filters does not differentiate the viruses sharply from the bacteria, since some of the latter may also be filtrable. Certain of the smaller bacteria, such as Dialister pneumosintes and the organisms of pleuropneumonia and agalactia, will pass through the coarser grades of filters. The slender, flexible spirochaetes may work their way through the pores, particularly when filtration is prolonged. Some species of bacteria have been shown to exist in a minute, filtrable form from which typical organisms may develop in subsequent cultures. These forms have been considered by some to be a phase in the life cycle of bacteria, and it has been suggested that

the viruses themselves may be merely filtrable forms of ordinary bacteria. There is little evidence, however, in support of such an hypothesis.

Ultrafiltration with collodion filters has been utilized by Elford as a means of determining the size of various viruses. Filtration through these membranes is not affected by extraneous factors to the same extent as that through the earthenware types. By variations in the proportions of an acetone solution of collodion and an ether-alcohol mixture, membranes can be made of graded porosities. The technique of preparing them is complicated, but the membranes obtained by each of the different mixtures have a uniform permeability.

Size.—Measurements of the viruses must be made indirectly, since their size precludes the possibility of observing them. Particles measuring less than ½ of the wave length of the light used to illuminate them cannot be precisely delineated. The wave lengths of the constituent rays of ordinary white light lie between 400 and 700μμ, and, therefore, particles smaller than 200 µµ cannot be resolved. The presence of much smaller objects may be recognized by dark-field illumination when the light is reflected, but the object seen is the diffraction image and not the actual particle. By utilizing short wave (ultraviolet) light and a special microscope constructed with a quartz optical system Barnard has obtained photographic images of some of the larger viruses, and has calculated their size from the magnification used. The figures obtained corresponded well with those determined by Elford with ultrafiltration methods. Other workers, on the contrary, estimating the size by ultrafiltration, centrifugalization, and diffusion methods have obtained varying results. Rivers and others point out, however, that, since virus suspensions contain extraneous protein from the host, the figures obtained may represent merely the size of protein particles upon which the virus is adsorbed. Some of the viruses are apparently almost as large as small bacteria, and, when treated with a mordant and stain (which increases the magnitude of small particles), are said to be just visible. Others are estimated to be almost as small as a single molecule of a complex protein such as haemoglobin, which is considered now to be approximately 5.5µµ. Frei has calculated, from the size of the necessary molecules, that a particle less than 2 to  $5\mu\mu$  in size cannot be a living body.

Cultivation,—A number of the viruses have been propagated upon artificial media containing susceptible tissue cells. Visible colonies are never formed, but the multiplication of the virus can be demonstrated by animal inoculation. Some of the viruses seem to require a medium in which there is actual proliferation of the tissue cells, whereas others need only the presence of living cells. It may be necessary to provide tissue from a species of animal which is susceptible to the particular virus under cultivation, and even a special tissue from such an animal may be required. Thus, according to Maitland, the virus of foot-and-mouth disease seems to grow only in a medium containing the lips, tongue, pads, or skin of embryo guinea pigs. A medium containing minced chick embryo with fresh plasma and Tyrode's solution is suitable for many of the viruses, as is also the choreo-allantoic membrane of the developing chick. See p. 854.

It is generally believed that propagation of the viruses does not occur in the absence of living cells. Eagles (1935), however, has recently reported the cultivation of vaccinia virus in a medium containing only fragmented cells, and concludes that intact living cells are not indispensable. He did not succeed in cultivating them when these cell granules were removed from the medium. Other investigators, using the same technique, have been unable to confirm his results, and the question remains open. The successful cultivation of a virus on a cell-free medium would be of the greatest importance in

the study of the viruses, and would prove beyond question that they are independent, living organisms, not enzyme-like substances derived from tissue cells. Some of the viruses appear to exist in the body only as intracellular parasites, and it seems most unlikely that these can ever be propagated on lifeless media. The tissue cells in some of these virus cultures show inclusion bodies similar to those found in the animal body

Viability.—The viruses are apparently destroyed by various physical and chemical agents, such as heat, ultraviolet light, and various disinfectants, in much the same way as are bacteria. They are very resistant to desiccation, however, and may remain viable outside the body for a long time, a fact which is important in the understanding of epidemiological and other problems. A majority of the viruses retain their viability over long periods in 50% glycerin, while the vegetative forms of bacteria are killed within a short time. Attempts have been made to prove whether the viruses are animate or inanimate by their reaction to various chemical substances. The question is still unanswered, but nothing has been discovered that is inconsistent with the belief that they are living, independent organisms.

Purification of virus emulsions.—Virus emulsions, whether obtained from the animal body or from virus cultures, contain of necessity a large amount of extraneous cellular elements and protein that is not entirely eliminated by filtration. These substances interfere seriously with the study of the viruses, and purification of such suspensions has been attempted by various methods. The virus may be adsorbed completely from a suspension by the addition of a small amount of kaolin. The virus can then be eluted by suspending it in a weak solution of ammonium hydroxide. After removal of the kaolin the pH of this suspension is adjusted. Other substances, such as animal charcoal, or aluminium hydroxide, are used also to adsorb the virus. However, these substances also adsorb a certain amount of the protein material, and the purified suspension is, therefore, not strictly protein-free. Ledingham (1935) has prepared suspensions of the elementary bodies of vaccinia by adding ether to the material. The cells are broken up, and the lipoid material is dissolved in the ether, which is then removed by centrifugalization. The viability of the virus is affected by the ether, however, and for infectivity tests he prepares the suspensions by filtration and centrifugalization.

Hughes, Parker and Rivers (1935) have found that the chemical composition of a purified preparation of the elementary bodies of vaccinia is compatible with the belief that the viruses are living organisms. The virus of mosaic disease of tobacco has been obtained in crystalline form which resists heating up to 90°C. It is infective, and is said to behave more like a self-propagating enzyme (Vinson, Stanley, 1935). See p. 200.

Inclusion bodies.—In many of the virus diseases peculiar structures called inclusion bodies may be seen within the cytoplasm or the nucleus of the injured cells. They are easily seen in sections stained with Giemsa's, Mann's or Castenada's stain, and their demonstration is of great diagnostic importance. Examples of cytoplasmic inclusions are the well known Negri bodies in rabies, the Guarnieri bodies in the epithelial cells in the lesions of small pox and vaccinia, the psittacosis bodies, and the Bollinger bodies of fowl pox. Acidophilic intranuclear inclusions are found in yellow fever, Rift Valley fever, herpes zoster and febrilis, chicken pox and a number of virus diseases of animals.

The nature of these bodies is still debated. Many investigators believe that they are aggregations or "colonies" of the virus, while others consider them to be merely the products of a cellular reaction in cells under the influence of a virus. The presence of the virus in some of these inclusions has been proven by animal inoculation, whereas other types of inclusion bodies have been found to be non-infective for animals and

presumably do not contain living virus. The precise nature of the inclusions obviously varies somewhat in the different virus diseases, but it may be assumed that protein elements from the host enter into the composition of all of them, and perhaps also virus particles, either living, or inactivated and therefore not demonstrable. The term "chlamydozoa" was applied to these inclusions in the belief that they were composed of virus particles surrounded by an "enveloping mantle." They were originally believed to be protozoa.

Elementary bodies.—The Bollinger bodies of fowl pox, when properly stained, are seen to be filled with large numbers of minute coccoid bodies lying in an amorphous matrix and surrounded by a lipoid capsule. These so-called elementary bodies (or Borrel bodies) are believed to be actual virus particles, although it is impossible to exclude the possibility that they consist of protein particles upon which the virus is adsorbed. They are almost at the limit of visibility, although their size is probably somewhat exaggerated by the staining methods used to demonstrate them. Ledingham has shown that these Borrel bodies are specifically agglutinated by immune sera. The Paschen corpuscles of variola and vaccinia, and similar elementary bodies occurring in psittacosis, are also believed to represent the actual virus.

Immunity.—After recovery from a virus disease the serum of man, and of experimentally infected animals, acquires the ability to neutralize the homologous virus. In a majority of the virus diseases the immunity produced is lasting (except dengue, herpes febrilis, common cold and perhaps influenza). The virus-neutralizing serum will protect animals from infection if injected along with or just prior to inoculation of the homologous virus, but it appears to have little or no effect after the development of symptoms. The immunity is specific for the particular virus concerned, and, in the case of foot-and-mouth disease, for the particular sub-type (A, C, or O) of the virus. Ledingham has obtained specific agglutination of suspensions of the Borrel bodies of fowl pox and the Paschen corpuscles of vaccinia with their homologous antisera. In these respects the action of the virus-neutralizing antibody appears to be analogous to the ordinary antibacterial antibody, yet there are differences which are hard to explain. The union between the anti-viral antibody and the virus is loose and can be easily dissociated. By simple mechanical manipulation, by dilution with physiological salt solution (within certain limits) or by cataphoresis a fully virulent virus can be recovered from a neutral mixture. Findlay (1936) has found that mixtures of Rift Valley fever virus and immune serum, apparently neutral in doses of 0.4 cc., are active in small doses (0.03 cc.) or by intranasal inoculation (possibly because the inoculum is small). Sabin (1935) believes that there is in fact no combination with or alteration of the virus itself, and that the antibody in a neutral mixture acts only upon the tissue cells, protecting them from invasion by the virus. These facts have some bearing upon the advisability of vaccination in the various viral diseases.

These observations bring up again the question of the nature of the virus. Those who argue that the viruses are enzyme-like substances derived from the cells of the host under some unknown stimulus believe that it is to these inanimate cell derivatives that antibodies are produced. The question of the nature of the stimulus is left open, however, and it is difficult to conceive of any inanimate stimulus adequate to explain the epidemiological and experimental facts of virus infections.

Adaptation.—Many of the viruses are pathogenic for a single species of animal, whereas other species are relatively immune. Some of the viruses have, nevertheless, a remarkable ability to adapt themselves to a different species of animal, and by this

biological modification some of their characteristics may be altered so that they no longer behave in the same way toward the original host. For example, the virus of small pox, after adaptation to the calf, produces vaccinia and not small pox when re-inoculated into man, yet the immunity produced protects against both diseases. Ledingham has shown that in the adaptation of variola virus to vaccinia virus there is some change in the antigen (as shown by agglutination reactions), although the viruses still retain some common antigenic properties. Other viruses have been artificially adapted to relatively insusceptible animals, frequently with the production of quite different pathological lesions, and a resulting attenuation in virulence for the original host.

# DISEASES DUE TO FILTRABLE VIRUSES

The filtrable viruses cause many of the diseases of plants, arthropods, fishes, birds, mammals, and man, possibly also of bacteria, if d'Herelle's concept of bacteriophage is correct. Some of the virus diseases (yellow fever, dengue, and phlebotomus fever) in man, and many in plants are transmitted by various species of arthropods. The transmission may be simply mechanical, or actual propagation may take place. In the latter case an "extrinsic incubation period" is necessary before an arthropod becomes infective, but, once infective, it remains so throughout its life. Findlay has suggested that some of these viruses may have originated as arthropod parasites and subsequently have become partially adapted to various species of vertebrate hosts.

All viruses are apparently intimately connected with the living cells of the host. Some of them exist as harmless saprophytes in certain tissues, yet may be pathogenic for other tissues of the same host. After recovery from an infection the virus may persist in the body for long periods. The virus of herpes febrilis may be present constantly in the mouth and in various tissues and produce only an occasional lesion on the lips. Some of the viruses stimulate cellular proliferation (common warts, various fowl tumors, etc.) whereas others produce necrosis (yellow fever, Rift Valley fever). Some of the viruses, such as that of foot-and-mouth disease, show a marked selectivity for one particular type of cell, whereas others parasitize the cells of various tissues.

Classification.—Attempts have been made to classify the viruses by their affinities for cells derived from the different embryonic layers. These affinities, however, may vary in different species of animals, and some of the viruses isolated from one type of tissue may be adapted artificially to another type. There is, nevertheless, a fairly well defined group affecting only cells derived from the ectoderm. In this group are the viruses of the common wart, molluscum contagiosum, and trachoma, which parasitize exclusively the cells of the skin or mucous membrane (the dermotropic viruses); the viruses of variola, vaccinia, varicella, herpes zoster and febrilis, which may affect the

nervous system as well as the skin; and the viruses of rabies, epidemic encephalitis (St. Louis type), poliomyelitis, and certain diseases of animals which affect the central nervous system (the *neurotropic viruses*). The viruses of Rift Valley fever and yellow fever may parasitize cells from all the embryonic layers; and from these viruses variant strains having a particular predilection for the central nervous system have been produced.

Preservation.—Most of the viruses can be kept active and virulent for months if preserved in 30 to 50% glycerin (preferably in physiological salt solution or a buffered phosphate solution) and kept at ice-box temperature or lower. (Ordinary pathogenic bacteria are killed or inhibited by the glycerin.) The virus of yellow fever is an exception, and is inactivated within a few days by the glycerin. The viruses are preserved best by drying the material in vacuo and storing it in the ice box. Sawyer preserves the virus of yellow fever by freezing the material (blood or ground up tissue) and drying it in vacuo over sulphuric acid.

Laboratory diagnosis of the virus diseases.—The presence of a filtrable virus in the body may be determined by the inoculation of blood, tissue or other material into animals belonging to a species known to be susceptible to the suspected virus. (See table, p. 24 for a list of suitable animals and methods of inoculation.) Some of the viruses are pathogenic only for monkeys, and others have been transmitted only to human volunteers. The symptoms and pathological lesions in the inoculated animals may be sufficiently distinctive to identify the virus, and the identification is confirmed if control animals receiving a known virus-neutralizing serum in conjunction with the unknown virus are protected. The demonstration of cytoplasmic or intranuclear inclusion bodies in smears or sections from the inoculated animals is also of importance in the diagnosis of the diseases in which they occur. In rabies the demonstration of the Negri bodies in the brain of the suspected dog as well as in that of inoculated animals is the most important diagnostic procedure.

When rabbits are used it is important to remember that they are subject to a spontaneous infection with a virus described by Rivers and known as virus III.

The presence of virus-neutralizing antibodies in the serum of patients may be utilized for diagnosis. The serum to be tested is mixed with a suspension of the known virus, and varying amounts are injected into a series of susceptible animals. Controls with a known normal and known immune serum should be made. Complement fixing, agglutinating and precipitating antibodies have been found in the serum in a number of the virus diseases, and when suitable antigens are generally available, their demonstration should become a useful diagnostic procedure.

Hypersensitiveness to the proteins in virus-containing material from buboes, inoculated mouse brain, and culture has been demonstrated in lymphogranuloma inguinale, and intradermal tests are of great value in diagnosis.

Serum treatment.—Immune sera have been used successfully in prophylaxis in a number of the virus diseases. The results of treatment, on the contrary, are unsatis-

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factory, as might be expected from the pathogenesis of these infections. Once the virus has become fixed to the tissue cells it is apparently protected from the action of the immune serum, and Ledingham has shown that this fixation may take place, in the case of vaccinia virus, within a few minutes after inoculation.

Vaccination.—It is claimed that immunity to virus diseases cannot be obtained by vaccination with a "killed" virus as is possible in the ordinary bacterial infections. Vaccination with living viruses, on the contrary, may produce a high degree of immunity, but it is necessary to use a virus which has been attenuated in some way without loss of its antigenic properties. This alteration may be accomplished sometimes, but not always, by repeated passages of the virus in another species of animal. Neutralization of the virus by the homologous antiviral serum, and attenuation by drying and by various chemicals have been used to produce a suitable vaccine. Carbolized or formolized vaccines have been used extensively in veterinary work. Park and Brodie have used a formolized suspension of poliomyelitis virus for vaccination in children, and have obtained excellent results as measured by the production of virus-neutralizing antibodies. The formolized "inactivated" suspensions had lost their infectivity for monkeys, but it is impossible to conclude with certainty whether the formalin had actually killed the virus or had merely attenuated it in some way so that it was no longer infective. It has been reported that a virulent virus has been obtained by cataphoresis from a formolized, apparently dead, suspension. Animals have been immunized by the injection of sublethal quantities, or by multiple intradermal inoculations, of a fully virulent virus, but these methods are not suitable for human immunization.

In vaccination with living viruses there is a potential danger which must be kept in mind, and only long experience with a given procedure can demonstrate conclusively its safety. This fact is also true of vaccination with viruses which have been rendered non-infective by various chemicals. Flexner concludes from his work on poliomyelitis that the killed virus is antigenically inert, and, therefore, any virus preparation that stimulates antibody formation must be regarded as living.

Smallpox, Alastrim and Vaccinia.—We now consider the Guarnieri bodies, which were first reported in 1892 from the cytoplasm of corneal cells of rabbits inoculated with smallpox, as representing the aggregations of very minute particles of filtrable virus material (Paschen bodies) forming a colony-like mass. These inclusions (Guarnieri bodies) are easily recognized microscopically and are eosinophilic. They are from 1 to  $20\mu$  in size, and the Paschen, or elementary, bodies are about  $0.25\mu$ . Similar inclusions occur in vaccinia and in alastrim. They were formerly considered protozoal in nature (Cytorrhyctes variolae) and grouped as Chlamydozoa. Under favorable conditions these viruses are filtrable.

A very mild form of smallpox which is known as alastrim occurs in certain tropical areas. The experience of the medical corps of the U. S. Navy, which indicated that large bodies of well vaccinated marines were perfectly protected in the midst of a Haitian epidemic, attests the identity of alastrim with smallpox. The virus is contained not only in the skin lesions but also in the nasal and buccal secretions, and the disease is communicable before the eruption appears.

It is generally accepted that vaccinia is smallpox permanently modified by passage through the calf. A disease of horses called grease, when inoculated into cows, also produces vaccinia. Monkeys are susceptible to both smallpox and vaccinia. Rabbits show a rather typical eruption when inoculated cutaneously with cowpox material but do not show characteristic lesions with smallpox material.

The incubation period of variola is usually about 12 days. Leake states that the most important diagnostic points, in the order of their importance are: the distribution of the eruption, the course of the disease, the individual lesions, and the inoculation tests.

Paul's test.—This is an inoculation test and, according to Leake, more useful in the diagnosis of smallpox than any other laboratory procedure. However, the test is negative in about 50% of the cases, chiefly early or atypical infections, and unless this fact is recognized, a negative test may give a dangerous sense of security.

The test is carried out as follows: the contents of a vesicle or pustule are inoculated into the cornea of a rabbit. After from 48 to 72 hours the eyeball is enucleated, fixed in strong sublimated alcohol, and examined for the characteristic whitish papules. Histological sections show typical Guarnieri bodies in the corneal tissue.

McKinnon's test.—Inoculate material from a lesion of the patient intradermally into a normal and into a vaccinated rabbit. If the material contains the virus of small-pox, a red swelling develops in the normal rabbit in about 4 days and disappears by the 12th day. The vaccinated rabbit shows no reaction, or only a red nodule that disappears rapidly.

Gordon has obtained agglutination, precipitation, and complement fixation reactions with antigens prepared by emulsifying the lesions, and an antivaccinia rabbit serum.

Vaccination performed in a proper manner with a potent vaccine will result in a reaction which may be classified under one of the following forms:

The primary reaction or vaccinia, which indicates an absence of immunity to smallpox. In this the zone of redness, rather narrow from the 3rd to the 7th day, begins to spread suddenly about 7 days after vaccination, and is broadest in 8 to 14 days, and then fades rapidly and disappears. The reaction is accompanied by vesicle formation.

The accelerated or vaccinoid reaction, which indicates partial immunity. In this the broadest redness occurs and passes in 3 to 7 days after vaccination. Vesicle formation occurs.

The immediate reaction or reaction of immunity, which indicates full protection against smallpox. In this the broadest redness occurs and passes in 8 to 72 hours after vaccination. A slight elevation of the skin can be felt by passing the finger lightly over the vaccinated area. Vesicle formation is usually absent. This reaction can be distinguished from an ordinary intradermal protein reaction by the fact that the latter is rapid and reaches its maximum in about ½ hour, whereas the reaction of immunity to smallpox does not reach its maximum until at least eight, and

usually more than 24 hours after vaccination. The protein reaction has faded before the reaction of immunity appears.

Technique of vaccination.—Leake considers the multiple pressure or prick method (acupuncture) probably the best method of vaccination. This consists of a shallow. tangential pricking of the skin with a needle through a drop of the vaccine covering an area not greater than 3 millimeters (1/8 inch) in diameter. There is little chance of accidental infection, and the cruption is typical. Acetone has been found more satisfactory than alcohol for cleansing without irritating the skin. The needle is not thrust into the skin, but is held parallel or tangent to it, the forefinger and middle finger of the right hand above the needle and the thumb below. The needle should point to the operator's left and across the arm so that the thumb of the operator does not hit the skin. The side of the needle is then pressed firmly and rapidly, with a perpendicular motion from the wrist, into the drop about 30 times in 5 seconds. The elasticity of the skin will pull a fraction of an inch of the epidermis over the point of the needle at each pressure so that the vaccine is carried into the deeper epithelium (cuboidal prickle-cell laver) where multiplication takes place most easily. The remaining vaccine can be wiped off with sterile gauze. No signs of bleeding will occur, and evidence of the punctures will fade out in less than 6 hours. Formerly, when only 6 pricks or punctures were advocated, the method was shown to give fewer "takes" than the scratch method. By the use of 30 pricks the percentage of "takes" has been made as high as in any other safe method. For primary vaccinations, where the mildest possible "take" is desired, and where other attempts will be made promptly if the first fails, the number of pricks may be reduced to 10 or even to 1.

The important points in vaccination are (1) to make a small superficial insertion, and (2) to keep the vaccine below freezing if possible.

The material for the vaccine is taken from the vesicles of inoculated calves not later than one week after inoculation. The most potent material is in the pulp at the base of the vesicle, not in the lymph that exudes from the vesicle. The pulp is ground up and mixed with an equal amount of glycerin. The calves are then autopsied to make sure that no other disease is present. The virus is later tested for tetanus bacilli, pus organisms, and the virus of foot-and-mouth disease. The test for tetanus is very important. Cultures are made anaerobically and incubated for at least 7 days and checked by inoculating mice if necessary. If found free from any harmful organisms, the vaccine is then tested on rabbits for potency.

Rivers has cultivated vaccinia virus in a medium of minced chick embryo and Tyrode's solution which, when injected intradermally in man, produces immunity to calf lymph vaccine without scarring or marked systemic reaction. Goodpasture and Buddingh have developed a practical method of producing vaccine virus by inoculating the chorio-allantoic membrane of the chick embryo.

After recovery from smallpox, alastrim or vaccinia, immune substances are present in the scrum, which has been shown to have virus-neutralizing, agglutinating, precipitating, and complement-fixing properties. An antiserum produced by one of these viruses has been found to be active on all of them. Serum from convalescent cases of smallpox and also anti-vaccinia serum have been used in the treatment of smallpox.

Varicella is presumably caused by a filtrable virus, although actual filtration has not been demonstrated. Inoculations of both man and monkeys with bacteria-free fluid from the vesicles, however, has produced the disease. Acidophilic intranuclear inclusions similar to those found in variola can be seen in the affected cells.

The relationship of the virus to that of herpes zoster is still controversial. Clinical observations on the association between herpes zoster and outbreaks of chicken pox have suggested that the etiological agents might be identical. Recent reports on the results of complement fixation tests in the two conditions support this view, but it is not generally accepted at present.

Herpes zoster is due to a virus which has not been shown to be filtrable. The disease has not been transmitted experimentally from one individual to another. Intranuclear cell inclusions can be demonstrated.

Lymphogranuloma Inguinale. This sixth venereal disease, as it is sometimes called, or climatic bubo, the usual tropical designation, is now recognized as due to a filtrable virus. A distinctive skin reaction (Frei's skin test) enables us to group under this infection various clinical states which may or may not show inguinal buboes. It is now known to be widely distributed in temperate regions as well as in tropical areas. In the U. S. the disease is chiefly noted in the colored population.

The Frei test has enabled us to group with climatic bubo in males, esthioméne (vulvar elephantiasis) and the genito-ano-rectal syndrome of females. Inguinal adenitis in the female is rare. In man the initial infection is usually of the coronal sulcus, and drains by the penile lymphatics into the inguinal glands. The intrapelyic glands are rarely involved in the male. In the female the intrapelvic glands are the ones usually involved, and this leads to perisalpingitis, perirectal infection, and subsequent rectal stricture. J. B. Vander Veer et al. (1935), in 47 cases, have noted 21 cases of rectal stricture—all in the female, and only 3 of these in the white race. The common location of the stricture was from 3 to 5 cm. from the external sphincter. In the Frei testing of prostitutes we may obtain positive reaction in the absence of clinical signs in a small percentage of cases. Text books have noted that climatic bubo appears without an initial lesion, as is recognized for chancroidal, syphilitic, or gonorrhoeal buboes, but the present view is that there is a primary sore, which may even resemble a chancre, or be so small as not to be detected. Mixed infections are common. In a urethral discharge, negative for gonococci, this infection should be suspected. In climatic bubo the incubation period is protracted, approximately 4 or 5 weeks, and the glandular enlargement rather painless. The affected glands show pockets of necrotic material, and the process extends widely to the deep as well as superficial inguinal glands. Slight fever and malaise are usually noted, or there may be protracted fever, weakness, and anaemia.

The Frei test.—The antigen is made by diluting material aspirated from buboes or an emulsion of infected mouse brain with 7 to 8 parts of saline. The mixture is heated at 60°C. for 90 minutes, and on the succeeding day for 1 hour at 60°C.

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The skin test is most marked after 24 to 48 hours and persists for 5 to 10 days. It ranges from redness, with or without induration, to vesicle formation. About o.r cc. is injected intracutaneously in the forearm—a saline control on the other side is advisable. The allergic state develops in from 2 to 4 weeks after the appearance of the bubo. Vander Veer considers that the Frei test is best read after 4 to 5 days. Non-specific reactions usually subside after this interval.

Mice are very susceptible and the virus may be passed from animal to animal by brain passage—with increasing virulence. Monkeys have been infected.

Mouse brain antigen.—Grace and Suskind (1936) have developed a method of standardizing mouse brain antigen so that it is as reliable as the Frei antigen. As noted above, the virulence of the virus increases on mouse brain passage and with this increase the antigenic power is enhanced. Mice weighing 20 Gm. were inoculated with a suspension of virus of a virulence sufficient to kill from 85 to 100% of the mice in from 5 to 7 days. The brain of such a dying mouse was emulsified in sterile saline in such an amount that the heated product produced a papule not less than 7 mm. in diameter in an infected man. Using the eighty-eighth passage of the virus, they found that 0.1 cc. of a 1 in 13 dilution gave a papule varying from 7 to 10 mm. in diameter. When unstandardized mouse brain antigen was first used, there were reports of reactions with the brains of normal mice as well as of those containing virus, but these reactions of normal mouse brain did not give rise to a papule exceeding 5 mm. The products of tissue disintegration, whether in normal mouse brain or non-specific pus, are capable of producing a reaction on intradermal inoculation, but not equal to that of a virus-containing material. There are many objections to lymphogranulomatous pus. (1) The varying antigenic power. (2) Only bacterially uncontaminated pus can be used. (3) The difficulty of obtaining a sufficient quantity of the specific pus for commercial distribution. (4) The possibility of the existence of other venereal infections.

Tamura (1934) reports cultivation of the virus, using Tyrode's solution with sterile animal tissue. Original cultures were made from filtrates from gland pus, and repeated transfers made successfully. He noted that the cultural antigen gave as marked reaction as that of Frei. He also used this heated antigen therapeutically.

It is usually stated that there is no specific treatment.

Foot-and-mouth Disease is a highly contagious disease of cattle, pigs, sheep and goats characterized by a vesicular eruption in the mouth and on the feet and udders. Occasionally man contracts the infection by contact with infected animals or by drinking raw milk containing the virus. It is of interest that the disease is the first one found to be caused by a filtrable virus (Löffler and Frosch,

The virus is present in the vesicles and in the saliva, excreta and milk. In the prevesicular stage it has been found in the blood. In some instances the virus has been demonstrated in animals some time after recovery from the disease.

The properties of the virus have been studied particularly in England and on the continent in recent years. It is the smallest virus known, the size being estimated at from  $8-12\mu\mu$ . It is easily destroyed by heat and does not withstand pasteurization. It is, however, resistant to desiccation, and for this reasion, in part, epidemics among cattle are extremely difficult to control. The virus has been cultivated in a medium containing the lips, tongue, pads or skin of embryo guinea pigs, but seems to require particular tissue from a susceptible species of animal (Maitland). Three types of the virus have been differentiated immunologically, O, A and C, and the immunity developed in an animal after recovery from infection due to one of these types does not extend to other types.

Hyperimmune serum prepared with these types has been used for prophylaxis and for treatment in cattle. Vaccination with formolized virus or serum-virus mixtures produces an active immunity and has been used to a limited extent in Europe.

Trachoma.—Trachoma is a form of granular conjunctivitis, the etiology of which is not clearly established. Inclusion bodies, described by Prowazek, are found in epithelial cells scraped from the lesions, and it has been assumed that the disease was caused by a filtrable virus. Whether these are analogous to the elementary bodies of known filtrable viruses or whether they are clumps of extremely small bacteria, or degenerated cellular fragments is open to question. Noguchi was able to infect the conjunctivae of monkeys with material containing inclusion bodies, but the disease has not been produced with filtered extracts.

The Koch-Weeks bacillus, and various other organisms, have been considered possible causes (see p. 97). More recently Noguchi has isolated a small, filtrable, Gram-negative bacillus, which he calls B. granulosis, from cases of trachoma, and has produced a condition resembling trachoma in monkeys by inoculation of the conjunctiva. Others have failed to confirm his work. It has been suggested that dietary deficiency may play a part.

Common Wart (Verruca vulgaris).—The epithelial cells of the common wart have been shown to contain inclusion bodies, and Kingery and Wile have obtained a filtrable virus which appears to be the etiological agent.

Herpes Febrilis (simplex) is caused by a filtrable virus which is constantly present in the local lesions and sometimes in other tissues, including the central nervous system. Intranuclear inclusion bodies can be demonstrated in the affected cells. The virus has been cultivated in a medium of minced rabbit testis, rabbit plasma and Tyrode's solution, and characteristic inclusion bodies have been described in the cells of these cultures.

In 1920 Löwenstein and Grüter showed that inoculation of the fluid from a herpetic vesicle into the cornea of rabbits produced a severe keratitis. They were able to propagate the virus indefinitely through a series of rabbits. After recovery these were found to be immune for 6 months or more. In some of these experimental animals an encephalitis developed, and the herpes virus could be demonstrated in the brain.

The herpes virus has been obtained from the brain in cases of epidemic encephalitis, and Levaditi and others believe that both diseases are caused by the same virus. This

view is not generally accepted, however, and seems to have been disproven in the case of the St. Louis epidemic. The herpes virus was not encountered in any of the cases, and that obtained was shown by Webster and Fite to be specific.

Epidemic Encephalitis.—Three clinical types of encephalitis have been recognized: The "lethargic" encephalitis (Type A) which followed the influenza epidemic of 1917 to 1918; the Japanese encephalitis (Type B) of 1924, and the St. Louis type of 1933. A filtrable virus has been recovered only from the last.

In the encephalitis lethargica described by von Economo and known as type  $\Lambda$  encephalitis, somnolence, slight or irregular fever, and ophthalmoplegia were noted in the initial attack, followed by parkinsonian syndromes and various psychical residuals. These sequelae at times followed the acute stages, but not infrequently developed after a symptom-free period of months or years. In the St. Louis epidemic high fever at the onset with severe headache and stiff neck were more characteristic, and the eye manifestations and the sequelae of the type  $\Lambda$  encephalitis were exceptional. The death rate for the type  $\Lambda$  is approximately 30%, and that for the St. Louis outbreak about 20%. Residual parkinsonian and psychic manifestations were not observed in the St. Louis epidemic.

Etiological agent.—In the St. Louis epidemic Muckenfuss, Armstrong, and Webster (1934) showed that a filtrable virus was the etiological factor by infecting rhesus monkeys with filtrates from human brain emulsions.

The brains of successfully inoculated monkeys showed changes similar to those observed at human autopsies—perivascular accumulations of cells and nerve cell degeneration in the brain and upper part of the cord. At the Rockefeller Institute this virus infected their special strain of Swiss albino mice. Intracranial inoculation of 1-1,000,000 dilution of mouse brain was usually successful. Intranasal instillation required a larger dose. By filtration experiments the virus particles were found to measure from 22 to 33µµ.

The virus was neutralized by the sera of convalescents from this epidemic but not by sera from recovered cases of lethargic encephalitis, poliomyelitis, Japanese encephalitis or Australian X disease. Cross immunization with herpes and equine encephalomyelitis was not found.

The other forms of epidemic encephalitis are probably due to specific filtrable viruses which are not neutralized by the serum of cases from the St. Louis epidemic.

Levaditi and others have supported the view that the virus of herpes febrilis is identical with that of lethargic encephalitis. It is true that the herpes virus has been isolated from an occasional encephalitis brain and that animal inoculation with herpes virus may produce lesions similar to those noted in encephalitis. However, the presence of herpes virus in the brain of encephalitis cases is rather to be regarded as a coincidence

since this virus is frequently present in the tissues of those having occasional attacks of herpes febrilis.

Epidemiology.—Leake, Musson and Chope (1934), in the St. Louis epidemic, noted a late summer and early fall incidence, whereas the winter season is the time of greatest incidence of encephalitis lethargica. The period of incubation probably varied from 9 to 14 days. Multiple cases in the same family were exceptional, even more so than in poliomyelitis. The possibility of transmission of the virus by water, milk, or mosquitoes was excluded by a thorough investigation. There was a striking increase in both incidence and mortality with increase in age. Only 13% occurred in children under 15 years of age. No sex or race predilection was noted.

Laboratory diagnosis.—Leukocyte counts of 15,000 to 20,000 have been reported in some cases, and normal or decreased counts in others. The spinal fluid was clear with an increase in lymphocytes (averaging 50 to 200 cells, but occasionally up to 1000). There was not much variation from the normal either in the sugar or globulin content.

Post-vaccinal Encephalitis.—Since 1922 many cases of encephalitis following vaccination, similar to the types of encephalitis which occasionally follow measles, smallpox, possibly other virus diseases, and antirabic immunization, have been reported. The greatest outbreaks have been reported in England and Holland, where infantile vaccination is not general. Most of the cases occur in children and young adults who have been vaccinated for the first time. Infants seem to be quite immune. In England the incidence has been estimated at less than one case in 33,000 vaccinations. The mortality is slightly more than 50%.

Symptoms begin seven to fourteen days after vaccination with headache and vomiting. Within a week fever, rigidity of limbs, paralyses, first flaccid, later spastic, and coma may follow. Recovery, even after paralyses have occurred, is usually complete in post-vaccinal encephalitis.

Scattered areas of demyelinization in the neighborhood of the blood vessels, found particularly in the cerebrum, mid-brain, and pons, also in the anterior horns of the cord, occur in all of these types of encephalitis and distinguish them from poliomyelitis and epidemic encephalitis.

There are three theories of the cause of post-vaccinal encephalitis: (1) that it is a manifestation of vaccinia, (2) that vaccination has activated some latent virus present in the body (although such a virus has never been isolated from a case), and (3) that an extraneous neurotropic virus has contaminated the vaccine lymph. Most authorities favor the second theory. Points against the first view are: (1) that the same type of encephalitis occurs after spontaneous infection with other virus diseases, and (2) that its appearance as a post-vaccinal complication is recent, although vaccination has been used for more than a century. Against the third theory is the fact that no particular strain of lymph can be implicated.

Recently Gordon and, independently, Hekman have treated post-vaccinal encephaitis with human anti-vaccinal serum with good results. A specific antiviral vaccinal

serum prepared from horses is available, but it has not been used in enough cases to determine its efficacy.

Poliomyelitis.—This disease is caused by a filtrable virus which has a particular predilection for the central nervous system.

Clinical picture.—The incubation period of poliomyelitis ranges from 3 to 14 (usually 7) days. The onset is usually abrupt, with fever, malaise, headache, and often gastro-intestinal disturbances. During this prodromal period, which lasts from 48 to 72 hours, there are no characteristic symptoms. Signs of meningeal irritation such as muscular twitchings, tremors, general unsteadiness, and stiffness of the neck and spine may appear. The deep reflexes may be increased at this stage. Diplopia and nystagmus may occur. Until the onset of the characteristic flaccid paralyses the diagnosis is difficult, particularly in the sporadic cases.

Abortive cases are recognized in which the prodromal symptoms are mild and are not followed by any evidence of nervous system involvement. Since there are no distinctive symptoms or signs, the nature of the illness is usually suspected only in the presence of an epidemic, or in the case of contacts with a known case of poliomyelitis. These cases far outnumber the paralytic cases and are of great epidemological importance.

The pathological lesions in the nervous system consist of localized areas of ganglion-cell destruction with perivascular and interstitial small cell infiltration of the leptomeninges and gray matter of the cord. Ledingham (1935) believes that this infiltration is a secondary response to injury of the nerve cells by the virus. The nervous system lesions are usually situated in the anterior horns of the spinal cord and in the bulb, and death may result from paralysis of the respiratory muscles. In rare instances the upper motor neurones may be involved to such an extent that a spastic instead of a flaccid paralysis develops. It is believed that at least some of the cases of Landry's paralysis are due to this virus, and Zinsser has produced a typical poliomyelitis in monkeys by inoculation with material from a human case. Children are much more susceptible than adults, but the disease may occur at any age.

Pathogenicity for animals.—A typical poliomyelitis with characteristic clinical and pathological findings can be produced in monkeys by intracranial, intravenous, intraperitoneal, subcutaneous, or intranasal inoculation of a filtrate obtained from the spinal cords of human cases. The minimal infective dose by intranasal inoculation is much smaller than by other routes. The infection may be transmitted serially from one monkey to another. The virus has not been demonstrated in the blood or in the cerebrospinal fluid of human cases. In experimentally infected monkeys, however, the virus is widely distributed in the early stages of the disease, and has been demonstrated in the blood, cerebrospinal fluid, lymph nodes, and nasopharynx. Other animals, with the possible exception of the rabbit, are apparently not susceptible. Brodie and Park (1935) have rendered mice susceptible to the virus by repeated X-ray exposures, and have then transmitted it serially through normal mice. After a few passages, however, the virus was lost.

Epideniology.—The disease is spread mainly by droplet infection from carriers and from the abortive cases. Amoss has shown that the infection may b acquired by contact with a case during the incubation period. The atrium is usually the upper respiratory tract, particularly the nasopharynx. There is much evidence to show that the virus penetrates the hairs of the olfactory cells and ascends by way of the axis

cylinders of the olfactory nerves directly to the brain, and thence to the medulla and cord. Aycock reported epidemics caused apparently by the ingestion of infected milk.

The virus of poliomyelitis passes through the Berkefeld V and N filters, but may be withheld by the W type. Its size is estimated to be  $50\mu\mu$  or less. It has not been cultivated on lifeless media; but Flexner and Noguchi, and later Long, Olitsky and Rhoads, obtained apparent multiplication of the virus in special media containing living cells. It is easily destroyed by heating to  $50^\circ$  or  $60^\circ$ C. for 20 to 30 minutes. It resists drying well, and may live for long periods in water or milk. It resists glycerination, and may remain viable for years in brain or cord tissue preserved in 50% glycerin and kept at  $4^\circ$ C. Different strains of the virus vary in their virulence for animals; a single strain observed by Flexner fluctuated inexplicably in virulence over a period of years. The virus isolated from a case of poliomyelitis during an epidemic in Melbourne is apparently a different strain as determined by protection experiments.

The virus has been found to be inactivated by various oxidizing agents, and by adrenalin and cortin. Jungeblut (1935) has shown that vitamin C (cevitamic acid), present in the adrenals and in the brain, neutralizes the virus. Furthermore, an amount of vitamin C approximately that occurring normally in the central nervous system suffices for the inactivation.

Laboratory diagnosis. Cerebrospinal fluid.—In the prodromal period of the disease there may be no abnormality in the fluid obtained by lumbar puncture. Occasionally the pressure is increased. With the advent of meningeal involvement, however, the character of the fluid changes. It is usually clear, or there may be a faint haziness by transmitted light. The cell count is increased, varying from 20 to 1000, or even 2000 cells per cu. mm. At first these cells may be predominantly polymorphonuclears, but after 24 or 36 hours these are replaced by lymphocytes. The globulin is increased; glucose and chlorides are normal. A delicate clot may form in the fluid. Stained films show no bacteria. The virus has not been demonstrated in spinal fluid by animal inoculation.

Blood.—During the early phase of the disease there is usually a moderate polymorphonuclear leukocytosis (15 to 30,000) with a reduction in the relative number of lymphocytes. The virus has not been found in the blood in human cases, although in the experimental disease in monkeys it has been demonstrated in very early cases.

Immunity.—An attack of poliomyelitis confers a lasting immunity, and the serum of an individual, or a monkey, after recovery possesses virus-neutralizing antibodies. Adults have a much greater resistance to the

disease than do children, and various investigators have found virus-neutralizing antibodies in a large proportion (about 75%) of urban adults with no history of the disease. Aycock and Kramer demonstrated them in 83% of a group of normal mothers and also in their new-born infants, whom they believe to have been passively immunized. These facts suggest that the relative insusceptibility of adults is due to immunization from sub-infective doses of the virus, or from an unrecognized abortive attack of the disease. One may assume, furthermore, that the virus is much more widely distributed than the number of recognized cases of poliomyelitis would indicate, and that the occurrence of epidemics may be associated with an enhancement in the virulence of the virus by repeated rapid passages through susceptible children.

Serum treatment is still in the experimental stage. Since the preparation of an antiserum from ordinary animals is difficult and uncertain, human serum has been utilized, either from convalescents or from individuals with a history of the disease. Since most adults have virus-neutralizing properties in the blood which have been shown to be as potent in some instances as those found in convalescent sera, parental or pooled normal serum has also been used. Intravenous or intramuscular injections of 50 to 100 cc. are given as early in the disease as possible, and repeated after 4 to 6 hours. Intrathecal injections have not given better results, and some authorities believe that the resulting inflammatory reaction of the meninges may be actually harmful.

It is impossible at present to evaluate the results of serum therapy. All investigators agree, however, that it is useless after paralyses have developed. Diagnosis in the preparalytic stage is difficult, particularly in the sporadic cases. During epidemics abortive cases are numerous, and there is no means of determining which cases may develop nervous system lesions. Under these conditions the value of serum treatment can only be demonstrated by a large and carefully controlled series of cases. Brodie and Park (1935) believe that the pathogenesis of the disease precludes the possibility of effective serum treatment. In monkeys large doses of serum have no curative value, even when given very shortly after intranasal or intracerebral injection of the virus.

Favorable results have been reported, however, from prophylactic doses of immune or parental serum during epidemics, but the passive immunity acquired in this way is transient. The incidence of the disease is so irregular and unpredictable that the results obtained require confirmation.

Vaccination.—Since Flexner, Amoss and Rhoads demonstrated that animals could be effectively immunized by the injection of an attenuated virus, by sublethal doses, or by multiple intradermal injections of an active virus, vaccination of human beings in this way has been tried. Kolmer (1935) uses a 4% suspension of the spinal cord of a monkey infected with a remote passage strain in a 1% solution of sodium ricinoleate. He believes that after repeated passages through monkeys the virus has lost its infectivity for man. The virus is further attenuated by sodium ricinoleate. Vaccination with this living virus results in the production of virus-neutralizing substances in the blood, and presumably in an effective immunity. These antibodies may appear within 96 hours after the first injection, and monkeys have been protected by vaccination during the incubation period of the disease. There is, however, no proof that

the virus-neutralizing power of a serum is an accurate measure of the immunity of the individual. The safety of the procedure has been questioned by Flexner and others, particularly in view of the inexplicable fluctuations in virulence of the virus.

Brodie and Park (1935) report the production of virus-neutralizing antibodies in children, equal to those present in convalescents, by injecting an inactivated vaccine prepared from a 10% monkey cord suspension in a 0.1% solution of formaldehyde.

Leake (1935) has reported 12 cases of poliomyelitis with a high mortality, occurring in children outside the epidemic areas, which followed vaccination. The distribution of the paralysis corresponded to the site of the injection, and Leake concluded that the infection was caused by the vaccine.

The duration of the immunity produced by vaccination is not known. The difficulty and expense involved in preparing the vaccine and in determining the susceptibility of individuals restrict the practicability of the procedure. Although vaccination of presumably susceptible children may prove to be of value during epidemics, the safety of the procedure is still questionable.

Armstrong and his associates (1936), by applying astringent solutions (such as 0.5% alum and 0.5% picric acid in physiological salt solution) to the nasal mucous membrane of monkeys, were able to protect the animals from infection by subsequent intranasal instillations of virus. This solution was tried extensively in man as a nasal spray during a recent epidemic in Birmingham, Ala., but the reduction in the incidence of the disease reported (Feb., 1937) was not considered sufficient to establish the practical value of the procedure.

Rabies.—Rabies is primarily a disease of dogs, wolves, cats and other carnivorous animals, but is communicable to man and domesticated animals through the salivary secretions of rabid animals. It is spread most commonly by bites, but infections have occurred from the licking of apparently normal skin by rabid dogs.

Epidemiologically, rabid dogs are almost exclusively the source of infection. Rabies has been eradicated from England by rigid quarantine of imported dogs. The saliva of the rabid dog may be infective five days before the onset of symptoms and remains so until the death of the animal. The first symptoms in the dog are change of disposition followed by excitability (even viciousness), and ending in paralysis and death within 10 'days. Death follows development of symptoms invariably in man, and almost invariably in the dog, but the disease does not always develop from the bite. Clothing may absorb the saliva. Cornwall reported a series of 423 persons bitten by known rabid dogs. Of these cases 148 developed rabies. Lacerated bites about the face, neck or upper extremities are the most serious. In man the period of incubation is from 2 weeks to 6 months, usually less than 6 weeks. The first symptoms are irritability and depression, with early difficulty in deglutition. These are succeeded by extreme restlessness and hyperaesthesia. The dread of drinking water (hydrophobia) is due to painful reflex spasms. The temperature ranges from 100° to 102°F. The stage of excitement lasts about 2 or 3 days and is followed by a paralytic stage which lasts a few hours and ends in death.

An unusual paralytic type of rabies in man and in cattle has been reported in Trinidad and also in South America. It has been shown to be transmitted by the bite of the vampire bat, *Desmodus rotundus* (Lima, 1934).

Rabies is caused by a neurotropic virus, filtrable through all grades of Berkefeld filters. Inclusion (Negri) bodies are practically always demonstrable within the cytoplasm of the cerebral cells, but it is not known whether these bodies represent aggregates of the virus or cellular substances formed in response to its presence, or (probably) both.

After inoculation the virus apparently travels slowly to the central nervous system by way of the axis cylinders of the peripheral nerves. It has never been demonstrated in the blood. In the dog (seldom in man), the virus is constantly present in the saliva, and is believed to reach the salivary glands by way of the nerves.

The virus can be preserved for months in 50% glycerin. If frozen and desiccated rapidly, it maintains its virulence, but if dried at room temperature, it loses its virulence

within a week. Marked differences in virulence occur in various strains of street virus, and may be produced by adapting it to different species of hosts, but all types are immunologically related.

Diagnosis.—The symptoms and death of a rabid dog are important points in diagnosis: therefore, the suspected animal should be kept under observation. If rabid, the dog will develop clinical evidence of the disease and die within 5 days. If it is killed prematurely the characteristic histological changes in the brain may not have developed sufficiently to permit a diagnosis to be made. When the animal dies, the head and several inches of the neck should be removed and sent to the nearest laboratory. The campus major (smear preparation) head may be packed in ice, or the brain can showing Negri bodies. A, Negri bodies; be removed, sectioned, and placed in equal B, inner bodies within the Negri bodies. parts of glycerin and water. (Sterilize the diluted glycerin by boiling and allow to

Fig. 41.-Two nerve cells of hippo-American Veterinary w.)

cool.) This will preserve the virus for months. The diagnosis is made by the demonstration of the inclusion (Negri) bodies in the cytoplasm of the cells in the brain. These are practically constantly present and are characteristic of rabies.

The Negri bodies were first described by Negri in 1903. They are round or oval bodies from 1 to 20µ in diameter, which are present within the cytoplasm of the cerebral cells. They may be found anywhere in the brain but are most numerous in the following areas, in order: (1) cornu Ammonis (hippocampus major), (2) region of fissure of Rolando (in the dog, crucial sulcus), or (3) cerebellum. In street rabies large forms from 18 to  $23\mu$  may be found, whereas in the nerve tissues of animals inoculated with "fixed" virus only minute forms, 0.5 $\mu$  or less, may be detected. These bodies have been found four to seven days before the onset of symptoms.

The Negri bodies may be demonstrated by staining smears of the grey matter of the brain by some Romanowsky method, preferably by the Giemsa stain. The smears are made by mashing a thin slice of the outer grey matter rich in ganglion cells with a cover glass against the slide. Afterward the cover glass is gently drawn along the slide. Impression films may be used. The smears are then stained in the usual way.

The following method of staining is also good. The smear is fixed in methyl alcohol for 2 or 3 minutes, washed with water, and covered with a stain made by adding 3 drops of saturated alcoholic solution of basic fuchsin to 10 cc. of distilled water and then adding 2 cc. of Löffler's methylene blue solution. The stain on the slide is steamed gently, washed with water, and dried.

Since the relation of the bodies to the nerve cells is more or less disturbed in making smears, examination of stained sections is preferable. Fix a bit of brain tissue for 5 to 7 hours in Zenker's fluid, wash and dehydrate in graded alcohols and chloroform as usual, embed in paraffin, and cut sections. These may be stained with Giemsa's stain. The Negri bodies are brought out as lilac-red bodies in the blue cytoplasm of the nerve cells. It is necessary to differentiate in 95% alcohol.

Mann's stain may be used for smears or sections. After fixation in methyl alcohol the slides are immersed in the stain for 5 minutes and washed in distilled water. After passing through graded alcohols including two changes of absolute alcohol they are cleared in xylol. Better definition can be obtained by staining for from 12 to 24 hours and differentiating with alkaline alcohol (absolute alcohol 30 cc., sodium hydroxide, 1% in absolute alcohol 5 cc.). After 5 minutes, wash in absolute alcohol and then in water which may be slightly acidified with acetic acid. Dehydrate and clear in xylol. The stain is prepared as follows: Methylene blue (Gruebler 00), 1% aqueous solution, 35 cc.; eosin (Gruebler BA), 1% 35 cc.; distilled water, 100 cc.

By the Lentz method, the  $3\mu$  sections, after removal of the paraffin, are flooded with absolute alcohol. They are then stained with a 0.5% solution of cosin in 60% alcohol for one minute. Wash in water and stain for one minute in Löffler's methylene blue. Wash them again in water. Apply Lugol's solution to the section for one minute and then differentiate alternately in methyl alcohol and water until the section is pink. After washing in water, stain again with Löffler's methylene blue for one half minute, then wash in water and dry carefully with filter paper. Now differentiate in alkaline alcohol (1 drop of a 5% solution NaOH in 30 cc. absolute alcohol) until the section is pink, then quickly differentiate in acid alcohol (1 drop 50% acetic acid in 30 cc. absolute alcohol) until a slight blue outline to the ganglion cells is obtained. Treat rapidly with absolute alcohol and xylol and mount in balsam. The Negri bodies show as light carmine-pink bodies on the light-blue ground of the ganglion cells. This method can be used for brain smears also.

In addition to examining for the Negri bodies one may inoculate a rabbit or guinea pig subdurally with a salt solution emulsion of the brain. If the virus of rabies is present the inoculated animal will develop symptoms within 3 weeks and Negri bodies can be demonstrated. This

procedure checks the microscopical diagnosis and may replace it when decomposition prevents the demonstration of Negri bodies. Contaminating organisms may be killed by the glycerin so that inoculations are possible. When, from advanced putrefaction or other causes, the Negri bodies cannot be found, the changes in the Gasserian ganglia may give a diagnosis. In typical lesions the ganglion cells are more or less completely destroyed and replaced by cells of other types.

Local treatment.—Thorough cauterization of the dog-bite wound with pure nitric acid (no other cautery is efficient), as soon as possible after the bite, is imperative, even when the Pasteur treatment can be given later. Immunization should be started immediately after the bite has occurred.

Pasteur treatment.—By subdural inoculation of rabbits in series the virulence of the virus for rabbits is finally so increased that the rabbits die in six days. It is impossible to increase further the virulence of the virus, which is then termed "fixed virus." The pathogenic power of this virus for other animals is also changed so that it is not apt to cause rabies if injected subcutaneously.

To attenuate this virus, the spinal cord of the rabbit is removed and dried over caustic potash at a temperature of 23°C. The cord is divided into segments about 1 inch in length. Drying for about 15 days seems entirely to destroy the virus. To prepare the material for prophylactic injections a small portion of the cord is emulsified with normal salt solution. By the usual American method the first subcutaneous injection is of a cord which has been desiccated for 8 days. The treatment is given daily for about 20 days. The immunity is "active," and the immunizing agent is a "vaccine." The activity of the virus can be preserved for about a month by glycerin and cold storage.

Other methods of treatment are:

- 1. The Harris method.—In this the brain and cord are frozen by means of CO<sub>2</sub> snow, ground up, and dried over H<sub>2</sub>SO<sub>4</sub> for about two days. The virulence of the virus is reduced one-half. The virus, if kept at o°C, retains the same potency for at least six months.
- 2. The Cumming method.—In this the brain is emulsified in saline and dialyzed with formaldehyde solution. The virus is so attenuated that intracranial inoculation does not produce rabies.
- 3. The  $H\ddot{o}gyes\ method$ .—The fresh virulent cord is injected but so diluted in strength that it acts as an attenuated virus.
- 4. Phenolized method.—Fermi, and more recently Semple, have used virus which has been inactivated by the application of strong (1% or 2%) phenol. Before injecting, the carbolized emulsion is diluted to preservative strength (0.5%). This method is now used extensively for man and for prophylactic immunization of dogs.

Antirabic scrum has been prepared by injecting sheep with emulsions of rabbit's cord and brain, at first intravenously, then subcutaneously. This scrum contains virus-neutralizing antibodies, but is valueless for treatment.

A post-vaccinal encephalitis, similar to that which occasionally follows vaccinia, may occur but is rare. McCoy found records of only three cases in a series of 20,000 treatments in this country.

Benign Lymphocytic Chorio-meningitis.—This type of meningitis is caused by a filtrable virus which was first isolated and described by Armstrong and Lillie in 1934. The disease is characterized by a short, benign course and by a lymphocytic pleocytosis in the cerebrospinal fluid.

Clinical picture.—The onset is acute with sudden, severe headache, stiffness of the neck and vomiting. There is moderate fever, lasting from a few days to three weeks and declining by lysis. Blurring of the optic discs and a positive Kernig's sign have been found in some of the cases. The reflexes are variable, but there are no paralyses. Transient pareses of the cranial motor nerves and respiratory disturbances have been reported, however. There may be a partial remission after the first or second day with subsequent recurrence. The total leukocyte count and differential count are usually normal. The virus is found in the cerebrospinal fluid, less often in the blood.

Complete recovery usually takes place in from two to seven weeks without sequelae. One fatal case with autopsy has been reported (Viets and Warren, 1937), but demonstration of the virus was not mentioned. We have observed a mild case with headache and slight fever of two days' duration.

Treatment is symptomatic. Lumbar puncture relieves the headache and nausea.

In many of the cases showing this clinical picture it has not been possible to obtain the virus or to demonstrate anti-viral antibodies after recovery, and these cases are due presumably to some other etiological agent.

Cerebrospinal fluid.—The pressure is usually increased. The fluid is generally clear, although it may be slightly turbid. The cells, which are almost entirely lymphocytes (90% or more), are increased in number from 50 to 2000 (average 250). The globulin is slightly increased, but the urea, sugar, and chlorides are within the normal range. The colloidal gold test shows a meningitic type of curve.

Historical.—Wallgren (1925) suggested that this clinical picture was an entity and proposed the name "acute aseptic meningitis."

Viets and Watts in 1929 and 1934 reported 14 cases of meningitis which presented this picture and could not be ascribed to any of the recognized types of infection.

In 1931 Dickens reported two cases which showed this syndrome. Other infections were excluded by a thorough study. He suggested that the etiological agent might be a virus.

In the St. Louis epidemic of encephalitis in 1933 Armstrong inoculated six monkeys serially with cerebrospinal fluid from a fatal case of what appeared from the scanty records to be the prevailing type of encephalitis. The sixth monkey, which had been inoculated twice with the so-called Freeman strain of the St. Louis encephalitis virus without developing symptoms, showed fever on the eighth day after inoculation. The

brain showed gross lesions (congestion and edema) like those caused by the common epidemic encephalitis strains. Lillie, however, found distinctive microscopic lesions. There was extensive lymphocytic infiltration of the meninges and choroid plexus and of the perivascular tissues of the brain vessels. These changes reappeared on subsequent transfers and are characteristic of acute lymphocytic chorio-meningitis. It is not certain whether this virus came from the human patient of from a monkey used in the transfer.

In 1935, while working with the viruses of equine encephalomyelitis and hog cholera, Traub recovered an infectious agent from apparently healthy white mice which was distinct from both, and was later shown to be identical with the viruses of Armstrong and Rivers.

In 1935 Rivers and Scott inoculated animals with cerebrospinal fluid taken from two men who showed the syndrome described and isolated a virus. Cross protection tests by Traub, Armstrong, and Rivers showed that their viruses were serologically identical.

In 1936 Findlay, Alcock and Stern in England reported recovering the virus from two human cases and from a mouse autopsied in 1934 and from one of 15 stocks tested.

The virus.—Experiments have shown that the etiological agent is a filtrable virus. Eosinophilic intranuclear inclusion bodies have been found by Traub in cells in the meninges and in glia cells of the cortex. It has been demonstrated in the brain, blood, cerebro-spinal fluid and urine of monkeys during the febrile period. It is present in the brain, blood, urine and nasal secretion of mice and guinea pigs, and may persist in the blood in mice for months after the animal has recovered. The virus is distinguished from that of the St. Louis type of encephalitis by cross neutralization tests and by the length of the incubation period, which is shorter in monkeys and longer in mice.

The virus is capable of passing through Seitz pads, Berkefeld V, N, and W candles, and collodion membranes with an average pore diameter of  $150\mu\mu$ . The size of the virus must be 70 to  $80\mu\mu$  or less. It has been found to retain its infectivity in 50% glycerin for at least 235 days. It can be kept frozen and dried without losing its infectivity.

Virus-neutralizing antibodies have been demonstrated in the sera of recovered cases, but this protective power develops only late in convalescence (after the second week).

Pathogenicity for animals.—Rhesus and cebus monkeys, mice and guinea pigs are susceptible to intracerebral inoculation. Guinea pigs and some strains of mice can be infected by contact. The portal of entry is probably the nose. White rats and rabbits have resisted infection.

Infection in monkeys.—Armstrong inoculated successfully one monkey intrathecally and another intravenously, intraperitoneally and intratracheally. On the fourth to the eighth day after inoculation the monkey develops a temperature of 40° to 41°C., which

falls by lysis after three to ten days. He refuses food and loses weight. He sits with the head drooping and eyes closed. If disturbed, he moves slowly and hesitatingly as though stiff. Most monkeys recover.

Recently Armstrong and Wooley recovered the virus from non-inoculated monkeys. Moreover, by means of protection tests they found that the sera of 5 out of 44 of their stock monkeys contained antibodies. It seems probable, therefore, that the disease may occur spontaneously in monkeys.

Infection in white mice.—Symptoms appear in white mice on the sixth or seventh days after intracerebral inoculation. They may sit apart from the others with their hair smooth or only slightly ruffled. When one is lifted by the tail, there is a series of abnormal, rapid, jerky movements. The hind legs are spastic and held out stiffly. Most of the animals die in convulsions in from one to three days after the onset. In addition to lymphocytic infiltration of the meninges and choroid plexus there is usually a virus pneumonia.

Traub (1936) showed that the virus was spread within a colony of white mice by direct contact, probably through the nasal mucosa. Only young mice showed definite symptoms. In older mice the virus was demonstrated by intracerebral inoculation of blood or tissue extracts into normal mice. After recovery they become immune to intracerebral inoculation of virus. Strains differ in susceptibility to contact infection. Wild mice can be so infected, though less readily. It is not certain whether the virus occurred primarily in mice, or was conveyed to them by infected caretakers. Both Rivers and Traub favor the second view.

Infection in guinea pigs.—Guinea pigs are as susceptible to subcutaneous or intranasal injections as to intracerebral inoculation. Symptoms appear after 8 days, and may be limited to a febrile reaction, or in the severer cases there may be loss of weight, dyspnoea, salivation, conjunctivitis and somnolence. The mortality varies with different strains of virus. The principal lesions consist of a virus pneumonia, and often a subendothelial infiltration of the heart, liver necroses, and meningitis. Animals which recover show a high degree of immunity, and the serum has virus-neutralizing power.

Laboratory diagnosis.—This depends upon demonstrating the characteristic changes in the cerebrospinal fluid, and by producing the disease in animals by injections of spinal fluid, either intracerebrally into several mice, or (preferably) subcutaneously into guinea pigs.

Influenza.—This disease is difficult to recognize during interepidemic periods on account of the lack of any distinctive physical signs. The onset is abrupt with a rapid rise of fever, headache, pains in the back and in the calf muscles, and a rather characteristic soreness of the eye muscles. There is a prostration which is often out of proportion to the other manifestations, and may be prolonged. There may be (rarely) an erythematous eruption. There is usually a leukopenia. Influenza, like measles, reduces the resistance to other infections, and secondary infections with the pneumococcus, streptococcus, and Pfeiffer's bacillus are common. In fatal cases death is due to these secondary invaders and rarely if ever to the influenza virus alone. In the great pandemics the cases occurring

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during the first outbreak are generally mild, whereas those in the succeeding outbreaks are attended with a higher mortality owing to the more frequent and severe secondary infections. This lowering of the resistance is indicated by the leukopenia and by the disappearance of the tuberculin reaction. The clinical picture in influenza is very similar to that of dengue and the dengue-like infections, and the diagnosis is particularly difficult in regions where these are prevalent.

Eliology.—The relationship of the influenza bacillus of Pfeiffer to this disease has been discussed on page 96. The evidence at present indicates that if this organism plays any part in the production of the disease it is purely a secondary one, and that a filtrable virus is the etiological agent.

In 1919 Nicolle and Lebailly obtained a disease in monkeys resembling human influenza by intranasal inoculation of filtered extracts of the blood and nasal secretions of influenza cases, but were unable to carry the infection through a second series of animals.

By using a susceptible animal, the ferret, Wilson, Smith, Andrewes and Laidlaw (1933) were able to produce a disease similar to influenza by intranasal inoculation of filtered nasal secretions from influenza patients. They succeeded in transmitting it serially in ferrets by intranasal instillation and by contact. No other method of inoculation produced the disease. In these animals the virus was demonstrated only in the nasal mucous membrane. It is not found in the blood either in the inoculated animals or in human cases of the disease. Serum from these animals after recovery, and from human convalescents, neutralized the virus.

Andrewes et al. (1934) also produced the infection in mice by intranasal inoculation. In these animals the virus caused a bronchopneumonia similar to that which frequently occurs in human influenza, and the virus was demonstrated in the lungs with or without secondary invaders. The disease in mice was frequently fatal. The virus from human cases was not pathogenic for mice until it had been established in ferrets, and, therefore, mice are not suitable for isolating the human virus. On the other hand, both mice and ferrets are susceptible to the virus of swine influenza. Anaesthetization apparently renders the animal more susceptible and predisposes to the development of an extensive pneumonia. The virus isolated by these investigators from human influenza was shown by cross immunity tests to be related to (but not identical with) the virus of swine influenza. According to Shope, an animal which has recovered from either human or swine influenza is immune to both, but its serum protects only against the homologous virus. Swine influenza resembles the human disease also in the association of a Gramnegative bacillus (Hemophilus influenzae suis) with the virus. Shope has shown that the typical severe disease in swine depends upon the combined activity of both the virus and the bacterium. The virus alone causes only an ill-defined, mild, transient infection which is often afebrile, and the bacillus alone produces no ill effects.

The observations of Andrewes, Laidlaw and Smith (1935) and of Shope, Francis and others (1936) have shown that the viruses obtained from recent cases of human influ-

enza, even from widely separated regions, are identical with one another, but differ immunologically from that of swine influenza. They suggest, however, that the swine influenza virus is identical with the human virus of the pandemic of 1918. The disease appeared for the first time in swine in the United States at the beginning of the pandemic, and probably was transmitted to swine from man. These observers have found that the serum of a large proportion of adults examined who may have been infected at that time (but not of children under 12) contains antiviral substances for swine influenza, but in many cases not for the present human strains. Protective power for the human virus now prevalent is common in children as well as in adults.

Immunity.—There is little or no natural immunity to influenza, as is shown by the enormous morbidity at the beginning of an epidemic. An attack of the discase produces some immunity. This has been regarded as only relative in degree and of short duration, because of the occurrence of a succession of outbreaks for two or three years after each of the great pandemics. If Shope's views are correct, however, the acquired immunity must be more substantial and enduring than has been believed. If further observations prove the existence of viruses which are antigenically distinct, this may explain the apparent lack of immunity indicated by the epidemiological studies.

Common Cold.—The weight of evidence at present strongly favors the view that colds are due to a filtrable virus.

In 1914, Kruse, and later Foster, Dochez and others, demonstrated that colds could be produced in man and monkeys by intranasal inoculation of the filtered nasal discharge from cases of acute coryza. Dochez, and others, found that the virus multiplied in chick embryo medium, and after many transfers they were again able to reproduce the disease by inoculation.

It may be assumed that human beings possess some natural immunity to the virus, and that infection takes place more easily when this is lowered by fatigue, exposure, etc.

Measles.—This disease is believed to be caused by a filtrable virus since typical measles has been transmitted to human volunteers by filtrates of the naso-pharyngeal secretions, and of the blood at the height of the disease. The ordinary laboratory animals are not susceptible, but an atypical form of the disease has been produced in monkeys and transmitted serially. After recovery they have been shown to be immune.

The serum of convalescents contains protective substances which are effective in preventing the disease when given within a few days (3 to 5) of the exposure. If given later the attack is modified and mild, yet produces an effective immunity. The serum is given in from 5 to 15 cc. quantities. Since almost all adults have had measles their blood may be used when convalescent serum is not available. Adult serum, however, is somewhat less effective and double quantities are recommended. The globulins in the normal human placenta have similar protective power, and a preparation of this has been marketed.

Epidemic Parotitis (mumps).—Wollstein has demonstrated a filtrable virus in the saliva of patients during the first 3 days of the disease (occasionally up to the 6th day) with which she produced suggestive lesions in the parotids and testicles of cats. Injection of the filtrate into the salivary ducts of monkeys caused a disease resembling mumps

which could be transmitted serially. The serum of recovered cases neutralized the virus. The virus was neutralized by the sera of convalescent cases of mumps.

Psittacosis.—Psittacosis is an epizootic disease of parrots which may be transmitted to human beings and, rarely, from one person to another.

While most of the parrot cases have been in imported pets, there now seems to be an endemic center in the U. S. in parrakeet-breeding aviaries. The disease in man is characterized by fever, pneumonic consolidation, and paralysis, and is often fatal. Studies made by Bedson, Krumwiede and others during the outbreak of the disease in 1929 and 1930 demonstrated that it was caused by a filtrable virus. The virus is present in the nasal secretion and faeces of animals, and infection in man is believed to be through the respiratory tract (Rivers). The disease can be transmitted to rabbits, guinea pigs, and mice by the inoculation of blood as well as by filtered extracts of the liver and spleen. The characteristic pathological lesions in these animals are focal necroses in the liver and spleen. Rivers has also produced a pneumonia in monkeys by intranasal or intratracheal inoculations.

Levinthal first described minute coccoid bodies  $(0.2-0.25\mu)$  in the cytoplasm of the reticulo-endothelial cells in the spleen, liver and exudates, and called them "psittacosis bodies." This observation has been amply confirmed, and they are believed to represent the virus. They can be demonstrated with Giemsa's stain or, preferably, with Castaneda's method, in which these elementary bodies stain bright blue while the cytoplasm of the cell takes a pink stain. Bedson, using tissue cultures of mouse spleen, has described a cycle of development of these elementary bodies.

Man and animals, after recovery from an attack, are usually immune. Some observers have found that the serum then shows protective properties, and that it may cause agglutination and complement fixation with suspensions of the psittacosis bodies.

Rivers has produced the disease in animals by the inoculation of filtered sputum from human cases, and suggests this as an aid in the diagnosis of obscure cases. Mice are inoculated intraperitoneally with 2 cc. of filtered sputum on three successive days. They usually die after seven to ten (or at times twenty) days. At autopsy they show focal necroses in the liver and spleen in which the coccoid bodies can usually be demonstrated. Incidental bacterial infections should be excluded by cultures. Extreme care must be used in handling infected animals, by wearing gloves, gowns and masks to avoid inhalation of the virus, since serious laboratory infections have been frequent.

### YELLOW FEVER

Four tropical diseases, plague, cholera, typhus fever, and yellow fever have been associated with more fear-inspiring, morale-destroying, and economic upsetting effects than even war and famine. Of these, yellow fever has certainly been the most important in the Western hemisphere. It is only since 1926 that we have had laboratory methods of differentiating this disease from bilious remittent fever, blackwater fever,

and epidemic jaundice, the three diseases with corresponding mortality which have most often been mistaken for yellow fever. In 1919 Noguchi, dealing with cases of epidemic jaundice, diagnosed as yellow fever by experienced clinicians, reported the cause of yellow fever as Leptospira icteroides. We now know that this spirochaete is the same as L. icterohaemorrhagiae. Again, as the result of mouse protection tests, we know that the virus of the disease in West Africa is the same as that of the New World, thus indicating that the disease was brought to America with slaves from Africa (in infected mosquitoes).

In 1900 an American Commission (Reed, Carroll, Lazear and Agramonte) proved that Sanarelli's *B. icteroides* was not the cause of yellow fever. Influenced by Carlos Findlay's insistence that the disease was transmitted by a mosquito, *Acdes aegypti*, and impressed with the fact that, following the first case of yellow fever in a community, subsequent ones in the epidemic do not appear for about 2 weeks ("extrinsic incubation" of Henry Carter), they conducted their classical experiments.

They demonstrated that A. aegypti (Stegomyia calopus), biting a yellow fever patient in the first three days of his disease, but not afterwards, became infected, but was unable to transfer the infection to a susceptible person until a period of about 12 days had elapsed from the time of the infecting feeding. They also demonstrated that the cause was a filtrable virus. Further experiments showed that contact with patients, or with materials soiled by patients (fomites), had no part in infection.

Clinical picture.—Classically, yellow fever starts abruptly, without prodromata, with a rapid rise of fever, congested face, and severe pains in the back and head. About the third day there is jaundice, black vomit and anuria. Soper (1934) reported an outbreak of yellow fever at Cambuci, Brazil, in which 89% of 227 residents who reported recent illness gave positive protection tests. There were only 13 clinically recognized cases, with 5 deaths. Prior to the appearance of the first fatal case, the physicians of the town reported 250 so-called "grippe" cases, without respiratory symptoms.

Etiology.—In 1903, a French Commission verified the report of the American Commission as to the filtrability of the virus, noting that while it passed through Berke.eld V and N filters, it would not pass the W filter. In 1928, Stokes, Bauer, and Hudson, studying yellow fever in West Africa, confirmed the work of the above noted commissions. In addition, the West Africa Yellow Fever Commission reported that Macacus rhesus monkeys could be successfully infected, either by inoculation of virus-containing blood or tissue emulsion, or by the bite of infected mosquitoes. The virus in the infected mosquitoes will not pass the filters if emulsified with salt solution, but by using 10 per cent serum this is accomplished. Antibodies appear in the infected monkeys by the third or fourth day, which neutralize the virus, and this probably explains the noninfectivity of yellow fever blood after the first three days. An extremely small quantity of virus will bring about infection (dilutions of blood of 1 to 1,000,000 and even of 1 to 1,000,000 have proven infectious).

Accidents in laboratory workers seem to indicate that the virus will pass through the intact skin, and experiments with monkeys suggest that

infection can take place through the conjunctiva. The virus is highly concentrated in the bodies of infected mosquitoes (1 to 1,000,000), and a single bite can transmit the disease. The virus will grow in tissue cultures. It has been found that the monkey may be infected by other species of Aedes and even by mosquitoes of other genera (see p. 582). The disease has also been transmitted experimentally by ticks (see p. 532). The virus may be preserved for a year or more by desiccation in vacuo. At 55°C. the virus is destroyed in 10 minutes, and a temperature of 24°C. to 30°C. causes loss of virulence in 48 hours. At  $-10^{\circ}$  to  $-15^{\circ}$  the virulence is maintained for several months. Unlike most filtrable viruses it is inactivated within a few days by 30% to 50% glycerin.

Intranuclear inclusion bodies have been found in the cells of the livers of monkeys dying of yellow fever, and reports have been made of their presence in the nerve cells of the spinal ganglia. These inclusion bodies have been found in the livers of about 25% of human cases, dying of yellow fever.

Immunity.—An attack of yellow fever, even if so slight as to be clinically unrecognizable, confers a lasting immunity. Adults seem as susceptible as children; there is no maturation phenomenon. In surveys to determine previous prevalence, we now use Theiler's mouse method, as modified by Sawyer and Lloyd (1931). If the virus is injected intracerebrally, all mice die. The present method is to inject 0.03 cc. of sterile starch solution intracerebrally to fix the virus. This is followed by the intraperitoneal injection of 0.2 cc. of a 10 per cent emulsion of the brain of a mouse dying of yellow fever, along with 0.4 cc. of the human serum to be tested. Six mice are similarly inoculated, and various controls with known positive and negative sera. At least 5 of the 6 control mice receiving normal serum should die of yellow fever encephalitis in from 5 to 10 days. This neurotropic virus is also found in the adrenals and the sciatic nerve.

Since the reliability of Theiler's method of demonstrating specific protective substances in the serum was established, it has been used extensively to study the distribution of the disease, particularly in South America. Such studies by Soper and others have shown that yellow fever is still endemic over a much wider area than had been realized, and that in a large proportion of human cases the infection causes a mild illness, the nature of which had not been recognized. The diagnosis has been confirmed by examination of liver removed postmortem by means of a viscerotome.

Jungle Yellow Fever.—Studies have also revealed that yellow fever occurs in endemic form as sporadic cases and occasionally in small epidemics in isolated out-lying districts over vast areas of Columbia and Brazil, in which Aedes aegypti is not found. The disease largely affected

men who were laborers in the jungle, sparing the women and children except when the families lived in or adjoining the jungle. This suggested that transmission must be brought about by species of mosquitoes which infest the jungles rather than the settlements, and it has been shown that many other species are at least potential transmitters.

In these districts the incidence of the disease in man seemed too small to furnish an adequate permanent reservoir of infection. Studies of the jungle fauna (Soper, 1935) showed that certain species of monkeys which are numerous there are highly susceptible to infection, and that in a substantial proportion of the monkeys examined protective antibodies were present in the blood. They, therefore, constitute a natural reservoir of infection. It is possible that the hedge hog, which is highly susceptible, may play a similar rôle in African yellow fever (Findlay et al., 1935). It has also been shown that certain other species which are not susceptible to the disease may, if subjected to infection, harbor the virus in their blood for some days (until antibodies develop). The practical significance of these mammalian reservoirs has not been determined, but they make the problem of prevention very difficult. The possibility that arthropods may serve as a permanent reservoir has also been suggested.

Vaccination.—Sawyer (1934) found that a vaccine prepared from neurotropic mousebrain virus and human immune serum was protective. Since the personnel of the International Health Division was vaccinated, no case of yellow fever has occurred, whereas Stokes, Noguchi, Young, Lewis, and Hayne died while investigating yellow fever.

Lloyd and Mahaffy (1935) have recommended the use of guinea pigs in testing for immunity, as less serum is required, but the method is less accurate (95%). Intracerebral inoculation of neurotropic mouse-brain virus into guinea pigs produces fatal encephalitis. Two animals are inoculated, each with 0.05 cc. mouse-brain virus (0.5%) and 0.15 cc. of the serum to be tested.

**Dengue.**—This disease is transmitted by certain species of *Aedes* (Stegomyia), in which the virus is propagated.

Its onset is more abrupt than that of any other disease. A short initial fever of three or four days is followed by a rapid fall, a short remission, and then a short terminal fever—saddle-back chart. The end of the primary fever is often characterized by critical sweats, diarrhoea, and epistaxis. The striking eruption, which has given it the name bouquet, appears about the time of the remission. Severe pains in back, joints, and postorbital muscles make the name given by Benjamin Rush appropriate—breakbone fever. The subsequent neurasthenia and physical weakness are very striking.

Arthropod vector.—The early investigations pointed to a common tropical mosquito, Culex fatigans, but subsequent experiments have shown that certain suspected species of Culex are incapable of transmitting the virus. Siler (1926) transmitted the disease by Aedes aegypti in 47 out of 111 human experiments. The cycle in the mosquito requires 11 to 14 days, and the infecting feeding must take place during the first 3, or possibly 5 days (Blanc) of the disease in man. Cleland and colleagues (1916) could not transmit the virus through C. fatigans but were successful with Aedes. The mosquito retains infecting power throughout her life—174 days (Blanc).

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Virus.—Virus-containing serum remains infective for 2 or 3 months if kept in sealed tubes and in the dark. The virus is destroyed by a temperature of 50°C. for 30 minutes. Blanc has found that desiccated and refrigerated serum would remain virulent only 95 days. It has been found possible to transmit the virus to monkeys, producing after five days of clinically negative infection, an infecting serum which will cause the disease in man, through transmission by Aedes fed on monkey blood.

Relationship to yellow fever.—Many authors have stressed the similarity of dengue and yellow fever clinically and epidemiologically, and there has arisen an idea that an attack of dengue might give a certain degree of immunity to yellow fever. It is generally accepted that the immunity following yellow fever is absolute and fairly permanent, while that from dengue is most variable and individual, pointing to several strains of dengue virus and a single strain of yellow fever virus. Dinger tried to infect mice with dengue virus by Theiler's intracerebral method of inoculation without effect. He subsequently inoculated a number of the same mice with yellow fever virus and all died of encephalitis. Snijders, Postmus, and Schuffner (1934) used the sera of 20 volunteers who had been experimentally inoculated with dengue virus, and using both the Theiler test and Sawyer's modification, they were unable to demonstrate any protective influence of these sera against yellow fever virus.

Epidemiology.—Blanc (1932) notes that for an epidemic the locality should have been free from outbreaks for a considerable period and that Aedes mosquitoes should abound. The explosive outbreak follows a less dramatic one, the virus being maintained chiefly in Aedes. Then at the time of most active breeding the widespread epidemic appears.

Rift Valley Fever (Enzootic hepatitis) is an epizootic disease of sheep, caused by a filtrable virus described by Daubney, Hudson and Garnham in 1931. During the study of a severe epidemic in Kenya, British East Africa, it was noted that the native shepherds, and later the Europeans, in contact with the affected animals developed a short illness resembling dengue. These investigators also produced the disease in a volunteer by inoculation with the blood of an infected sheep. Since that time a number of laboratory workers in this country and abroad have contracted the disease while studying the virus.

The disease can be transmitted to cattle (a small epidemic of natural infection is recorded), goats, certain species of monkeys, rats, mice and other small rodents. Guinea pigs and rabbits, however, appear to be resistant. The infection can be produced by application of the virus to scarified skin, the conjunctivae, or nasal mucosa, as well as by subcutaneous or intraperitoneal injection. There is some evidence to suggest that mosquitoes of the genus *Mansonia* act as vectors, but this has not been definitely proven. The characteristic pathological lesion in lambs, and also in inoculated mice, is a marked focal necrosis of the liver. Stained sections show acidophilic intranuclear inclusions in the hepatic cells.

#### FILTRABLE VIRUSES

Mackenzie and Findlay (1936) obtained a "fixed" neurotropic strain of Rift Valley fever virus by the injection of immune serum into an animal prior to an intracerebral inoculation of virus. Some of the nerve cells showed intranuclear inclusions similar to those produced by the neurotropic type of yellow fever virus. They suggest that this restraining action of specific antibodies may produce neurotropic variants of other viruses, and that the occurrence of nervous sequelae in virus infections in man may be explained in a similar way. The virus occurs in the blood, liver, spleen and other organs early in the disease in both animals and man. It will pass through Berkefeld candles N, V, and W; and Broom and Findlay have estimated its size at from 23 to  $35\mu\mu$  by filtration through collodion membranes. Mackenzie has cultivated the virus in a medium of chick embryo and Tyrode's solution. It can be preserved in blood containing 0.5% phenol for months in the ice chest.

Immunity.—The serum of convalescent animals and man contains virus-neutralizing antibodies for several months after recovery, but the immunity is not permanent. Broom and Findlay have demonstrated complement-fixing antibodies which are apparently specific for Rift Valley fever, and which they found to persist for at least 6 months. Francis and Magill (1935) have shown that the inoculation of insusceptible animals such as the rabbit also results in the development of virus-neutralizing antibodies.

The diagnosis of Rift Valley fever depends upon the demonstration of the virus in the blood by mouse inoculation in early cases, or of virus-neutralizing antibodies later in the disease. The clinical picture resembles that of influenza, dengue, or yellow fever. The virus of influenza, however, is not present in the blood; and the viruses of dengue and yellow fever are not pathogenic for mice on intraperitoneal inoculation. There is no cross immunity with the viruses of dengue or yellow fever.

Phlebotomus Fever (Pappataci fever, Sandfly fever).—This disease resembles dengue, influenza, and similar infections, but it is of short duration—usually only 3 days. It is due to a filtrable virus which is present in the blood during the first 24 to 48 hours. It is transmitted by a moth midge or sandfly, Phlebotomus papatasi (possibly other species also), in which a developmental period (extrinsic incubation) of from 6 to 8 days must elapse before it becomes infective. The virus has been shown to be transmitted to a second generation of these flies, which apparently constitute a reservoir of the infection. See p. 562.

#### BACTERIOPHAGE

Bacteriophage is a filtrable, yet particulate substance which causes lysis, or dissolution of susceptible bacteria. It increases in quantity in the process, and can be transmitted indefinitely from one culture to another.

Twort, in 1915, while working on the filtrable viruses, observed a "glassy degeneration" of certain colonies of cocci in which no intact organisms were demonstrable in smears. Inoculation from these areas into normal colonies produced similar areas. This lytic agent was filtrable and could be transmitted serially in cultures. In 1917 d'Herelle discovered that sterile filtrates from the faeces of a case of Shiga bacillus dysentery contained a substance which would, after a preliminary period of incubation (4-24 hrs.), inhibit and finally dissolve the organisms in an actively growing culture of the Shiga bacillus. He also found that filtrates from this lysed culture acted similarly

on a fresh culture even in minute amounts, and that the lytic principle could be propagated indefinitely in Shiga bacillus cultures.

The nature of this lytic substance is still disputed. D'Herelle, who has been the most active investigator of the problem, is convinced that it is a minute organism living as a parasite on susceptible Lacteria and has named it Protobios bacteriophagus. Many other investigators, however, believe that the phenomenon is due to autolytic ferments derived from the bacteria themselves. Bordet and his associates postulate the theory that a mutational change, which he terms a "hereditary nutritional vitiation," occurs in the bacteria. These variant strains then develop the property of producing active lytic substances, which in turn affect other sensitive bacteria. In order to explain the development of lysogenic strains (which contain the lytic principle but are themselves resistant) he further assumes that such strains continue to produce the lytic agent and transmit this property to other generations, yet are themselves able to resist the lytic action. Hadley has made the suggestion that the bacteriophage is perhaps a filtrable phase in the life cycle of the bacteria.

Demonstration in cultures.—Bacteriophage can be obtained easily from faeces or sewage. It is often present in water and soil and has been isolated from pus, infected urine, and other substances. It is sometimes present in laboratory cultures in a latent or even in an active form. After isolation by filtration its potency can be increased by repeated inoculation into susceptible young cultures. Old or dead cultures are not affected. It is never demonstrable except in conjunction with actively growing young cultures.

Its presence may be demonstrated in either fluid or solid cultures. Inoculation of a suitable broth culture causes a partial or complete clearing of the turbidity in from 4 to 24 hours. The potency of the bacteriophage preparation can be measured by adding varying amounts to a fresh culture. In some cases as little as one part of the filtrate in a billion parts of culture will effect lysis. If transplants of such a mixture are made to an agar plate shortly after inoculation, a surface growth is obtained which is pitted with clear glassy areas or plaques. These plaques may occur in the center or around the edges of discrete colonies, giving the culture a motheaten appearance. Smears from these areas show only an amorphous debris. When lysis becomes complete, no growth is obtained in subcultures. D'Herelle refers to these plaques as colonies of bacteriophage.

The growth of bacteriophage in a culture frequently results in the development of variant types of the culture, which may be enhanced or diminished in virulence. S forms may be changed to R forms, and mucoid types frequently develop.

Mechanism of lysis.—Under the microscope the affected bacteria can be seen to swell to a relatively great size and burst suddenly. D'Herelle believes that this phenomenon is due to multiplication of the bacteriophage within the cell. Bronfenbrenner has suggested that the decomposition of the proteins of the cells by some ferment or enzyme may raise the osmotic pressure and cause water to be absorbed into the cell.

Viability.—Bacteriophage may withstand heating to 75°C. for ½ hour and resists drying for some time. According to d'Herelle its resistance in general is greater than that of vegetative bacteria and less than that of sporebearers.

Specificity.—Bacteriophage is generally (though not always) specific for certain species of bacteria. It may act upon closely related species. Sometimes its activity is limited to a certain strain of a species. Adaptation to other organisms may occur to some extent. D'Herelle believes, however, that there is only one very adaptable bacteriophage, while others believe that there are distinct types. It has been found to be antigenic. Since the bacteriophage cannot be obtained free from lysed bacterial bodies, antisera contain also ordinary bacterial antibodies. After absorption of such sera with the bacteria themselves, however, a substance remains which will inhibit the action of the homologous bacteriophage. This antibody is said to be specific for the bacteriophage used in its production, irrespective of the bacterial substrate upon which the bacteriophage was propagated.

Use in therapy.—D'Herelle believes that bacteriophage action plays a significant part in recovery from infection, and in the rise and fall of epidemics. He claims to treat successfully such infections as dysentery, typhoid, plague, cholera, etc. with bacteriophage preparations. These results have not been confirmed by others. Such solutions are very antigenic by reason of the lysed bacteria which they contain, and this fact must be remembered in evaluating the results obtained.

A number of authors, however, report good results from its use locally and parenterally in localized septic infections, and also in colon bacillus infections of the urinary tract. Krestownikowa and Gubin have demonstrated that when bacteriophage preparations are injected parenterally, the lytic agent is found in all the tissues within a few minutes, but disappears within 6 or 8 hours.

#### PROTEIN NATURE OF VIRUSES

By using the ultracentrifuge, which gives a force roo times that of the ordinary centrifuge, the individual molecules of the more complex, heavy proteins can be sedimented from a solution. Wycoff (1937), by applying this method to the study of mosaic diseases of tobacco and potatoes and of rabbit papilloma, obtained concentrated proteins which reproduced these diseases and which were many times as infectious per unit of weight as the material from which they were derived. There was a parallelism between the rate of disintegration of these proteins and the loss of infectivity. These observations indicate that the particles of these viruses are associated with, and possibly are, individual protein molecules.

Stanley found that the infectivity of tobacco mosaic virus could be destroyed without loss of its antigenic properties. This fact is of tremendous import if it proves to be true of other viruses. The rate of sedimentation of staphylococcus bacteriophage indicates a molecular weight of over 50.000.000.

### CHAPTER X

# BACTERIOLOGY OF WATER AND MILK

#### WATER

BACTERIOLOGICAL examinations are used to determine whether or not water from a given source is fit for human consumption. The isolation from water of such intestinal pathogens as the typhoid, dysentery, or cholera bacillus is extremely difficult, because their number is usually very small in proportion to the quantity which can be cultured. Such cultures, therefore, have no value as a test of the safety of a particular water supply. In practice the possibility of their presence is inferred whenever sewage contamination is demonstrated. Judgment is based on (1) a count of all viable bacteria present in the water, and (2) a test for organisms from sewage. In the United States the detection of bacteria of the coli-aerogenes group is accepted as the best evidence of sewage contamina-English authorities also consider sewage streptococci and the spore-bearing B. enteritidis sporogenes of value as indicators of such pollution. The presence of the former shows recent pollution, and that of the latter also indicates contamination, which, however, is not necessarily recent or serious, if E. coli and streptococci are absent.

Enumeration of Bacteria.—Glass-stoppered bottles with a capacity of 25 to 100 cc. are used. If they cannot be sterilized by the usual method, they may be washed with sulphuric acid and rinsed before collection. If the specimen comes from city water supplies, let the water run for a few minutes before collection. If it comes from a pond, stream, or cistern, dip the bottle at least 10 inches below the surface before removing the stopper with forceps. The fingers must not touch the stopper or the neck of the bottle in filling. Stirring up sediment increases the count. The specimen should be packed in ice until analysis. The interval before examination should not exceed 6 hours for impure water or 12 for pure water.

Technique.—If the quality of the water is unknown prepare dilutions up to 1:1000 or, higher. Put 1 cc. amounts of the undiluted water and of each dilution into Petri dishes, making duplicate sets. Add 10 cc. of lactose litmus agar (melted and cooled to 40°C.) to each dish in one set, mix, and incubate at 37°C. for 24 hours. Add 10 cc. of nutrient gelatin (similarly melted and cooled), or else agar, to each dish in the other set, mix and incubate at 20° for 48 hours.

Make counts in the plates which show from 25 to 250 colonies and multiply by the dilution factor. The average represents the number of viable bacteria per cc. originally present.

If there should be very many colonies on a plate, it will be found that counting is facilitated if the surface be marked off into segments with a blue pencil. If colonies are very numerous, cut out of a piece of paper an aperture 1 cm. square. Count the number of colonies visible through this aperture when applied to different parts of the plate, and strike an average for each square observed. To find the number of such spaces contained in the plate, multiply the square of the radius of the plate in centimeters by 3.1416. Then multiply this number by the average per square centimeter,

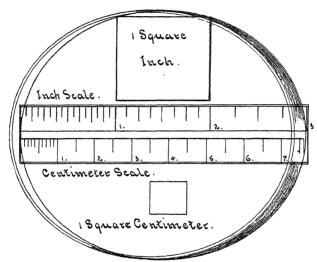


FIG. 42.—Estimating colonies on water plate. The diameter of the bottom of this Petri dish is 3 inches or 7.5 + centimeters. The area of a circle is equal to the square of the radius multiplied by  $\pi$  or  $^{2}$ /7.  $_{1}$ /2 in. = radius.  $_{1}$ /2  $\times$   $_{1}$ /2 = 2.25.  $\times$  2/2/7 = 7.07 square inches. 3.75 cm. = radius. 3.75  $\times$  3.75 = 14.06. 14.06  $\times$  2/2/7 = 44.1 square centimeters. Number of bacterial colonies in 1 sq. in. averages, approximately, 75. Number in 7.07 sq. in. = 530. Number of bacterial colonies in 1 sq. cm. averages, approximately, 12. Number in 44.1 sq. cm. = 528.

and we have the total number of colonies on the plate. This is the principle of the Jeffers disc.

Bacteria which normally live in the intestinal tract naturally grow best at body temperature, whereas those which normally occur in water prefer the average temperature of lakes and rivers. Consequently, when the number of bacteria growing at 37°C. approaches the number growing at 20°, sewage organisms must be suspected. Good water shows a ratio of from 1:25 to 1:50. That of polluted water may be as high as from 1:4 to 1:2. There is no generally accepted standard as to the maximum count

permissible in potable water. A count higher than 500 colonies per cc. on plates incubated 24 hours at 37°C. may be regarded as excessive. Some have set the limit at 100 colonies per cc.

Since water containing sewage streptococci or bacilli of the *coliaerogenes* group shows pink colonies on the lactose litmus agar plates, whereas good water does not, their appearance points to faecal contamination.

It is recommended that all Gram-negative, non-spore-forming bacilli which ferment lactose with gas production and which grow aerobically on standard media be included in the *coli-aerogenes* group.

Identification of Members of the Coli-aerogenes Group. 1. The presumptive test.—Inoculate a series of lactose broth Durham fermentation tubes with graduated quantities of the water, not more than 10 cc. in a tube (10 cc., 1.0 cc., 0.1 cc., etc.). The quantity of the medium must be at least twice that of the water. Incubate these tubes at 37°C. for 48 hours. Examine them at 24 and 48 hours and record gas formation.

The formation within 24 hours of gas occupying more than 10% of the inverted vial constitutes a positive presumptive test.

If the gas formed in 24 hours occupies less than 10% of the inverted vial or if none is seen before 48 hours, the test is doubtful and must be confirmed.

The absence of gas after 48 hours constitutes a negative test. (The time limit of 48 hours doubtless excludes from consideration occasional members of the *coli-aerogenes* group which form gas very slowly, but for a standard test their exclusion is considered immaterial.)

2. The partially confirmed test.—Make two or more Endo or eosin methylene blue plates from the tube which contains the smallest amount of water showing gas. Make the transfers as soon as possible after the formation of gas. Incubate the plates at 37°C. for from 18 to 24 hours.

If typical colonies develop in this period, the partially confirmed test may be considered positive.

If, however, no typical colonics appear, the test is not definitely negative, since members of the group may have failed to form typical colonies or may have grown slowly. The test must be completed.

3. The completed test.—From the Endo or eosin methylene blue plates transfer two or more typical colonics, each to an agar slant and lactose broth fermentation tube. If no typical colonics have appeared after 24 hours, incubate for another 24 hours. Then two or more of the colonies most likely to belong to the group, whether typical or not, are transferred to agar slants and lactose broth fermentation tubes.

The inoculated tubes are incubated until the formation of gas or for 48 hours. The agar slants are incubated for 24 hours. Then at least one culture, corresponding to a tube which shows gas formation, is examined microscopically.

The formation of gas in lactose broth and the demonstration of Gram-negative, non-sporing bacilli in the agar slants are complete proof of the presence of a member of the *coli-aerogenes* group.

The absence of gas formation or failure to demonstrate such bacilli constitutes a negative test.

In order that these tests may show the number per cc. (or per 100 cc.), at least three portions of each sample should be used. The largest amount should give a positive and the smallest a negative test. When examining potable water, at least 5 tubes should be inoculated with 10 cc. of water. The presence of organisms of the colon group in more than one (or more than 10%) of the 10 cc. samples examined may be regarded as evidence of contamination.

Tests have been suggested for differentiating faecal from non-faecal members of the group, but they are not regarded as dependable.

The following tests have been employed for differentiation of the colon and aerogenes groups.

Methyl red test.—The peptone medium is described on page 842. Inoculate 10 cc. portions of this medium and incubate at 37°C. for 4 days. Use 5 cc. of the culture for this test and 5 cc. for the Voges-Proskauer reaction.

The methyl red indicator solution is prepared by dissolving o.r gram methyl red in 300 cc. alcohol and diluting with 200 cc. distilled water. Add five drops of this solution to 5 cc. of each culture. A distinct red color is recorded as methyl red +, a distinct yellow color as methyl red -, and intermediate colors as ?.

Voges-Proskauer reaction (Acetyl-methyl-carbinol reaction).—Fill fermentation tubes with a 2% glucose Dunham's peptone solution, sterilize and inoculate. Incubate for three days and then add 2 or 3 cc. of strong potassium hydroxide solution. The development of a pink color on exposure to the air is a positive reaction.

Sodium citrate test.—Dissolve 1.5 Gm. sodium ammonium phosphate (microcosmic salt), 1 Gm. potassium dihydrogen phosphate, 0.2 Gm. magnesium sulphate, and 2.5 to 3 Gm. sodium citrate (crystals) in 1 liter distilled water. Incubate cultures for 4 days at  $37^{\circ}$ C. and record growth as + or -.

The typical reactions for organisms of the colon group are: M.R. +, V.P. -, and citrate -. Those for members of the aerogenes group are: M.R. -, V.P. +, and citrate +.

The Coli-aerogenes Group.—The diagnostic characteristics considered important by the American authorities in identifying the colon bacillus are:

- I. Typical morphology; non-sporing bacillus; relatively small and often quite thick.
- 2. Motility in young broth cultures. (This is at times unsatisfactory, as some strains of the colon bacillus do not show it even in young broth cultures.)
- 3. Gas formula in dextrose broth. Of about 50% of gas produced, one-third should be absorbed by a 2% solution of sodium hydrate  $(CO_2)$ . The remaining gas is hydrogen. (Later views indicate that the gas formula is exceedingly variable and should not be depended upon. To carry out this test one fills the bulb of a fermentation tube with the caustic soda solution, holding the thumb over the opening, or inserting a rubber stopper, the broth culture and the soda solution are mixed by tilting the fermentation tube to and fro. The total amount of gas is first recorded and then that remaining after the  $CO_2$  has been absorbed is reported as hydrogen.)
  - 4. Non-liquefaction of gelatin.
  - 5. Fermentation of lactose with gas production.
  - Indol production.
  - 7. Reduction of nitrates to nitrites.

To these may be added the acidification and coagulation of litmus milk without subsequent digestion of the casein. The production of gas and fluorescence in glucose neutral red broth is also a very constant function of the colon bacillus. A. aerogenes is similar to E. coli with the exception of non-motility, formation of gas in starch media (bubbles on potato slant) and frequent lack of indol production. It is often, especially in milk cultures, provided with a capsule and rarely forms chains. It resembles the Friedländer bacillus but differs from the typical pneumobacillus by producing acid and gas in lactose broth and by its coagulation of milk.

B. coli anaerogenes also is similar to E. coli but does not produce gas in glucose and lactose. This organism is not usually recognized by American authorities, but I have found on Endo plates an organism showing a red colony with metallic luster which failed to produce gas in either glucose or lactose.

Note.—The reduction of neutral red with a greenish-yellow fluorescence is very striking and has been suggested as a test for the colon bacillus. Many other organisms, especially those of the hog cholera group, have this power. It is convenient, however, to color glucose broth with about 1% of a 0.5% solution of neutral red.

On the plates made for the detection of colon bacillus certain organisms may be found which originate from faecal contamination. The more important of these are those of the paratyphoid, cloacae and proteus groups. In addition, the Alcaligines faecalis has not rarely been isolated. Among natural water bacteria there may be present either the liquefying or the non-liquefying B. fluorescens. These colonies have a yellowish-green fluorescence.

Certain chromogenic cocci and bacilli are found in uncontaminated waters as B. indicus or B. violuceus. From surface washings we obtain certain soil bacteria as B. mycoides, B. subtilis, B. mcgatherium. One of the higher bacteria which shows long threads, Cladothrix dichotoma, is common, and is characterized by a brown halo around its gelatin-plate colony.

Freezing kills most of the bacteria in water. Less than 1% of typhoid organisms inoculated into water survive a week of freezing. Ice that has been stored for 4 or 5 months is bacterium-free.

A commission composed of eminent American bacteriologists and sanitarians have recommended the following as maximum limits of bacteriological impurity:

- 1. The total number of bacteria developing in standard agar plates, incubated twenty-four hours at 37°C., shall not exceed 100 per cc.: Provided that the estimate shall be made from not less than two plates, showing such numbers and distribution of colonies as to indicate that the estimate is reliable and accurate.
- 2. Not more than one out of five 10-cc. portions of any sample examined shall show the presence of organisms of the *coli-aerogenes* group.
- 3. It is recommended, as a routine procedure, that in addition to five ro-cc. portions, one r-cc. portion, and one o.r-cc. portion of each sample examined be planted in a lactose-peptone broth fermentation

tube, in order to demonstrate more fully the extent of pollution in grossly polluted samples.

Isolation of the Typhoid Bacillus from Water.—This is probably the most discouraging procedure which can be taken up in a laboratory. Owing to the long period of incubation of the disease, the typhoid organisms may have died out before the outbreak of an epidemic suggests the examination of the water supply.

There have been various methods proposed for the detection of the *E. typhosa* in water. A method which would offer about as reasonable a chance of success as any other would be to pass 2 or 3 liters of the water through a Berkefeld filter; then to take up in a small quantity of water all the bacteria held back by the filter. Then plate out on Endo's medium and examine colonies which do not show any pink coloration. The dysentery bacillus has about the same cultural characteristics as the typhoid so that it is important to note motility. If from such a colony you obtain an organism giving the cultural characteristics of *E. typhosa*, carry out agglutination and preferably bacteriolytic tests as well. Some strains of typhoid, especially when recently isolated from the body, do not show agglutination.

Isolation of the Cholera Vibrio from Water.—The method proposed by Koch in 1893 does not seem to have been bettered by later investigators. Add 1% of peptone and 1% of salt to 100 cc. of the suspected water and incubate at 38°C. At intervals of 8, 12, and 18 hours examine microscopically loopfuls taken from the surface. When comma-shaped organisms are seen, plate out on agar. Colonies showing morphologically characteristic organisms should be tested for agglutination and bacteriolysis. Since the cholera vibrio shows a marked cholera-red reaction, it is advisable to inoculate a tube of peptone solution from such a colony, incubate for 18 hours, and add a drop of concentrated sulphuric acid. The rose-pink coloration is given only by the cholera vibrio with the acid. The organism produces the nitroso factor in the reaction.

Examination for Streptococci.—Add varying amounts of the water to a 1% glucosebroth medium and incubate for 48 hours. Examine hanging-drop preparations for streptococci.

Examination for Cl. welchii.—Add 50 cc. of the water to 100 cc. of sterile milk. Heat the flask at 80°C. for 15 minutes and pour sterile liquid paraffin on the surface to preserve anaerobiosis. The appearance after incubation of the "stormy-clot" reaction indicates the presence of Cl. welchii.

#### MILK

In examining milk, counts of the total number of bacteria are of value chiefly as an indication of the care used in handling the milk. Tests for specific pathogenic organisms, on the other hand, may be very important. Anderson has found that top milk contains from 10 to 500 times as many bacteria as bottom milk. Cream separated centrifugally contains more than that raised by gravity.

The specimen should be representative of the lot from which it is taken, and the milk in the container should be mixed thoroughly before the collection. Each specimen must

include at least 10 cc. It should be kept in a sterile, glass-stoppered bottle on ice until the examination can be made. Always shake a specimen thoroughly before removing a portion.

Methods of Quantitative Examination. 1. The standard plate count.—Prepare dilutions of 1:100, 1:1000, and 1:10,000 or higher, according to the probable impurity of the milk. Incubate 1 cc. of each in 10 cc. of nutrient agar, melted and cooled to 40°C., for 48 hours at 37°C. Count the colonies on the plates which contain from 30 to 300 and calculate the average number of viable bacteria per cc.

2. The microscopic count (the Breed method).—This method, which is convenient for immediate use, makes it possible to count also bacteria which have died or been unable to grow. It can, therefore, be used for preserved specimens. On the other hand, there is a large factor of error.

Smear 0.01 cc. of the milk on a slide over an area of 1 sq. cm. Dry the film in a warm place, avoiding excessive heat. Immerse the slide for  $2\frac{1}{2}$  minutes in a Coplin jar filled with xylol to remove fat, drain, and rinse in acetone for 2 or 3 minutes. Stain the slide in a fresh aqueous solution of methylene blue until it is overstained. Next wash it in water and decolorize in alcohol until only a faint blue tint remains. Dry and examine microscopically. By using the  $\frac{1}{12}$ -inch objective and No. 5 ocular and adjusting the length of the draw tube so that the diameter of the field is exactly 0.2 mm. (four small squares of the haemacytometer ruling), the area of the field is made just  $\frac{1}{3183}$  sq. mm. Find the average count of a large number of fields and multiply this by 3000, rather than 3183. Since only 0.01 cc. is on the slide, the product is multiplied by 100 to find the number of bacteria per cc. (See the section on The Microscope.)

The reductase test (or methylene blue test).—This is based upon the fact that the color given to milk by a small quantity of methylene blue disappears at a rate depending almost entirely upon the oxygen consumption of the bacteria.

Specially prepared tablets of methylene blue (National Aniline Co.) are recommended for preparing the reagent, but a suitable stock solution may be prepared with a 1:20,000 dilution of the dry dye. It is advisable to heat the solution to 100°C. a few minutes before use.

Place 10 cc. of milk in a test tube and stir 1 cc. of the reagent through it. The color is a robin's egg blue. Leave the milk in a water bath at 37°C. or in an incubator, observing it every 15 minutes, until the milk regains its normal color. In certain European countries where the test is widely used the color of the upper fourth of the tube is disregarded, especially if it disappears unevenly.

An exact agreement between the standard plate count and the interpretation of the reduction time cannot be expected. Undoubtedly bacteria affect this test which are missed by the culture method. There is every reason to believe that it detects bad milk as accurately as any other method. The test is useful when laboratory facilities or trained assistants are not available or when speed is necessary.

The following standards are used in Scandinavian countries:

Class 1. Good milk.—Not decolorized in 5½ hours. It contains as a rule less than a half million bacteria per cc. (according to the plate method).

Class 2. Milk of fair average quality.—Decolorized in less than 5½ but not in less than 2 hours. It contains as a rule from one-half to four million bacteria per cc.

Class 3. Bad milk.—Decolorized in less than 2 hours but not in less than 20 minutes. It contains as a rule from four to twenty millions per cc.

Class 4. Very bad milk.—Decolorized in 20 minutes or less. It contains as a rule twenty million or more bacteria per cc.

The British Ministry of Health has laid down the following standards for "graded" milks:

Certified milk.—Viable bacteria not to exceed 30,000 per cc.; E. coli to be absent from 0.1 cc.

Grade A milk.—Viable bacteria not to exceed 200,000 per cc.; E. coli to be absent from 0.01 cc.

Grade A pasteurized.—Same standard as for certified milk.

Ordinary pasteurized milk.—Viable bacteria not to exceed 100,000 per cc.

# Qualitative examinations

E. coli.—Make dilutions of the milk up to 1:100,000. Add varying amounts of the dilutions to tubes of lactose-bile-salt medium. Find the smallest quantity which shows production of acid and gas.

The presence of liquefying organisms, demonstrated on gelatin plates, is evidence of probable contamination by faecal bacteria.

Tubercle bacilli.—Two 50 cc. amounts of the sample are centrifuged at the rate of 3000 revolutions a minute for a half-hour. Suspend the sediment in each tube in 3 cc. of sterile salt solution. Inject two guinea pigs subcutaneously on the inner side of the thigh with 3 cc. One pig is autopsied after four weeks and the other after eight, if the first shows no tuberculous lesions. It is necessary to use two pigs, since one may die from another infection.

Certain other acid-fast bacteria, such as the butter bacillus, may also cause lesions. The simplest method of distinguishing them from tuberculous lesions is to inoculate the animals with 2 cc. of old tuberculin when they are ready to be tested. If tuberculous, they will die within a day. Glycerin agar may be inoculated with material from the lesions and incubated at 37°C. Other bacteria usually show colonies within a few days, whereas tubercle bacilli do not.

- E. typhosa (and the Salmonella).—Centrifuge part of the specimen and inoculate the cream and sediment into eosin methylene blue plates and broth. Incubate the rest and make frequent transfers. Study selected colonies for identification.
- C. diphtheriae.—Culture by inoculating blood agar plates with a mixture of cream and sediment.

Streptococci.—Haemolytic streptococci, usually of the alpha type, are found in even the cleanest milk. It has been suggested that those pathogenic for man are probably all derived from human sources. They are cultured for study like diphtheria bacilli.

Br. abortus.—Inoculate two guinea pigs as in the test for tubercle bacilli. After the autopsies cultures are made from the spleen and studied.

Agglutination of Br. abortus by the serum of the guinea pig is sufficient proof that the milk was infected.

# CHAPTER XI

# STUDY AND CLASSIFICATION OF MOULDS

CLASSIFICATION OF FUNGI PATHOGENIC FOR MAN

Class	Family	Genus	Species
Phycomycetes  Ascomycetes	Mucoraceae	Absidia	A. corymbifera
		Mucor	M. mucedo
		Rhizopus	R. niger R. parasiticus
	Ashbyaceae	Piedraia	P. hortai
	Coccidioideaceae	(Coccidioides	( P. colombiana C. immitis
		Rhinosporidium	R. seeberi
		(Histoplasma	H. capsulatum
	Eremascaceae	Zymonema	Z. dermatitidis (Crypto-
			coccus gilchristi) Z. capsu <sup>1</sup> atum
	Eremascaceae Imperfectae	Castellania	C. tropicalis
			C. pt. mona'ls
			S. albicans
		Syringospora	S. psilosis
	Saccharomycetaceae		S. interdigitalis  C. histolyticus
	Imperfectae	Cryptococcus	C. meningitidis
	Trichophytoneae	Malassezia	M. furfur
		<b>6</b> 0.1.1.1	T. tonsurans
		Trichophyton	T. sabouraudi
		Megatrichophyton Ectotrichophyton	M. roseum E. mentagrophytes
		Microsporum	M. audouini
		and out of our	A. schoenleini
Fungi Imperfecti		Achorion	A. muris
			E. interdigitale
		Epidermophyton	E. floccosum
	Aspergillaceae	Endodermophyton (Aspergillus	E. concentricum A. fumigatus
		Penicillium	P. bertai
		Scopulariopsis	S. brevicaulis
		Allescheria	Λ. boydii
	Toruleae	(Dematium	D. wernecki
		Madurella   Indiella	M. mycetomi
		(Indiena	I. brumpti A. bovis
	Actinomyceteae	Actinomyces	A. madurae
			A. asteroides
			A. minutissimus
	Sporotrichieae	∫Sporotrichum	A. thuillieri
			S. schencki S. beurmanni
		Aleurisma	A. albiciscans

## GENERAL CONSIDERATIONS OF PLANT PARASITES

The vegetable kingdom is divided into the following phyla: Myxophyta or slime moulds, Schizophyta or fission fungi, Thallophyta, embracing red, green, and brown algae, fungi and lichens, Bryophyta or mosses and liverworts, Pteridophyta or ferns, and the Spermatophyta, the seed plants.

The only botanical phyla which concern us in human parasitism are the Schizophyta and the Thallophyta.

The classes Schizomycetes and Schizophyceae belong to the phylum Schizophyta. In the class Schizomycetes we have the order of Eubacteriales, which embraces such important families as Spirillaceae, Coccaceae, Bacillaceae and Bacteriaceae, which are considered in the preceding chapters. To the class Schizophyceae belong the blue-green algae (Cyanophyceae), which do not concern us in human parasitology.

In the phylum Thallophyta we have the red, green, and brown algae, fungi and lichens. Lichens are fungi symbiotic with or parasitic upon algae. In Thallophyta there is no distinct differentiation into root, stem and leaf. The plant body may consist of one cell or many cells forming the thallus. Only the fungi are of medical importance.

- Nomenclature.—The rules governing zoological nomenclature are discussed in Chapter XVI (Animal Parasitology), page 391. In Bergey's "Determinative Bacteriology" (fourth edition) and in Dodge's "Medical Mycology" will be found a discussion of the rules of botanical nomenclature considered by an International Congress in 1930 at Cambridge, England. Botanical nomenclature corresponds largely to that of zoology, but differs in some details. The following points apply particularly to botanical nomenclature.
- (a) To avoid disadvantageous changes in the nomenclature of genera by the strict application of the rules and especially of the principle of priority, a list of names is provided which must be retained in all cases. These names are, by preference, those which have come into general use in the fifty years following their publication.
- (b) Orders are designated preferably by the name of one of the principal families with the ending "ales."

Suborders have the ending "ineae."

Families are designated by the name of one of their genera or ancient generic names with the ending "aceae."

Subfamilies have the ending "oideae"; tribes, "eae"; and subtribes, "inae."

- (c) The generic names are governed by the same rules as in zoological nomenclature.
- (d) The specific name should, in general, give some indication of the appearance, the characters, the origin, the history, or the properties of the species. If taken from the name of a person, that of the one discovering, or describing, or concerned with it should be given.
- (e) Specific names begin with a small letter except those taken from names of persons. (The custom in zoological nomenclature is to use a small letter for the specific name, even if it is that of a person. Bergey has followed the zoological rule.)
- (f) No one is authorized to reject, change or modify a name because it is badly chosen or disagreeable, or because another is preferable or better known, or because of the existence of an earlier homonym which is universally regarded as non-valid.

MYCOSES 2II

#### Fungi

The following classes of thallophytes are of medical interest: (1) Phycomycetes, (2) Ascomycetes and (3) Hyphomycetes or Fungi Imperfecti. Fungi, like bacteria, do not have chlorophyll, hence they must live a saprophytic or parasitic existence. In their simplest form they are ramifying filaments called hyphae. A network made up of vegetative hyphae, intertwined in tangled threads, is termed the mycelium. Growth may be either by addition of new hyphae (apical growth) or by division in a single hypha (intercalary growth). The hypha may be a single cell or many cells separated by septa.

Some hyphae show cellulose, others chitin. In some fungi the mycelium becomes packed as hard masses, containing food material to serve future germination, and known as sclerotia.

Probably only in the case of *Piedraia hortai* are such structures present in pathogenic fungi, but in the mycetomas the granules are of this nature, and known as bulbils. Ergot is the sclerotium of a fungus (*Claviceps purpurea*) attacking the grain-bearing heads of rye, and is of importance medicinally and as a food poison. In the Middle Ages there were great epidemics of ergotism, causing gangrene (St. Anthony's fire).

Spores.—Equally with the hyphae are the spores (conidia) important structures. The simplest definition of a spore is a cell which may or may not separate from the hypha, but is capable of germinating and reproducing the parent cell or hypha. A spore may be non-sexually produced by simple separation from the hypha (conidium), development along the course of the hypha (oidium or chlamydospore), or within the protoplasm of the hypha (endospore). We usually have in mind, when referring to a chlamydospore, a rather thick walled asexual spore, which is capable of resisting an unfavorable environment and, subsequently, germinating. Hyphae may separate from parent hyphae and form arthrospores (thallospores), which may subsequently become oval or round (blastospores). Blastospores, characteristically, sprout from hyphae.

Certain fungi have hyphae which form fruiting branches called conidiophores (sporophores). Where we have cells which give rise to endogenous spores (sporangium) the sporophore is called a sporangiophore.

In the class Ascomycetes we have as characteristic the ascus, a sporangium in which spores develop usually to form eight ascospores.

Where reproduction is of a sexual type we may have the gametes (sexual cells) equal or unequal, or the female gamete may be much larger than the male one (sperm), the latter fertilizing the egg which later produces oospores. Parthenogenesis is frequently present among fungi.

Mycoscs.—Diseases caused by fungi are known as mycoses. The terminology of the mycoses varies with different authors, but fairly well accepted are the dermatomycoses, or ringworm affections, otomycoses, onychomycoses and maduromycoses. Again, the name may be taken from the cause, as sporotrichosis, blastomycosis, actinomycosis or aspergillosis.

Pleamorphism.—Particularly in the ringworm fungi there are remarkable variations in pure cultures; thus the morphology of the periphery of a dermatophyte may be entirely different from the original central inoculation growth, and the change hold for subcultures from the peripheral zone. Also, different spore forms may develop from the same culture. This is usually referred to as pleomorphism, but the term polymorphic is more applicable to many of these variations. It is well recognized that variations may result from culture environment—hence the necessity for standardi-

zation of composition, temperature and reaction of culture media used in research. Another term used in mycology is saltation, where parts of the colony show differences. Some confuse mutation with saltation.

#### PHYCOMYCETES

Phycomycetes are sometimes called Lower Fungi, as more approaching primitive forms—the Ascomycetes and Hyphomycetes belonging to the group of Higher Fungi. The mycoses caused by Phycomycetes are of little importance, unless we follow the classifications of Brumpt and Ramsbottom where two important fungi, Rhinosporidium seeberi and Coccidioides immitis, are classified under Chitridiales, an order of Phycomycetes.

Otherwise, we have only to consider the Mucoraceae, which are moulds so often observed as cotton-like areas on decaying foodstuffs, or as Petri dish contaminants. The best known species is Mucor mucedo, so common on horse dung, but it is now denied that this fungus plays any part in human mycoses, although a case of mucor keratomycosis has been reported, where this common mould was isolated from a corneal lesion.

Absidia corymbifera (Mucor corymbifera).—The sporangium of this fungus averages from 50 to  $70\mu$  in diameter, with smaller ones from 10 to  $20\mu$ . The spores are spherical and measure from 3 to  $4\mu$  in diameter. It grows well on Sabouraud's medium. This species has been reported as the cause of pulmonary and aural mycoses (but very rarely).

Rhizopus.—Two species of this genus, R. niger and R. parasiticus, are, possibly, pathogenic, the former isolated from rare cases of "black tongue," and the latter from the sputum of a case diagnosed as pulmonary mycosis. Vuillemin doubts the relationship to black tongue. The columella of R. niger, after the dehiscence of the sporangium, is mushroom shaped. The sporangia, when mature, have a black color.

#### ASCOMYCETES

Among the Higher Fungi we have the classes Ascomycetes and Fungi Imperfecti. Ascomycetes are probably the most definitely characterized of the fungi, this class having a special type of sporangium called an ascus (little sac). In the young ascus we have a single nucleus, which by three successive divisions results in eight uninucleated ascospores. In some forms we may have only two or four ascospores—or even sixteen may be noted.

In this class we have the yeasts which reproduce typically by budding, but produce ascospores. Other budding forms may fail to show ascospore formation (asporogenous yeasts). In some Ascomycetes we have a well developed thallus, with septate hyphae (the hyphae of Phycomycetes are non-septate). In the yeasts only a sprout mycelium is known.

Trichosporosis.—In the involvement of the hairs, known as "Piedra," we have small nodules forming along the shaft, about the size of a head louse nit, but surrounding the hair, and not projecting off at an angle. These little masses are black and very hard,

hence the name piedra (stone). The infection is attributed to the use by women of a mucilaginous paste on their hair. It has also been reported from the hair of beards. The cause is *Piedraia colombiana* (*Trichosporum giganteum*).

Another species, *P. hortai*, is found in Brazil. Treated with potassium hydrate the nodule is found to be made up of polygonal cells, 12 to 14µ in diameter surrounding the hair shaft, but not invading it. It grows on Sabouraud's medium (better on carrot). Trichonocardiases, similar to piedra, have been reported by Chalmers, but caused by a Nocardia (Actinomyces). Similar masses, involving either the hairs of the head or bearded regions, have a wide distribution, according to MacLeod.

Coccidioidal Granuloma.—The first case, affecting a Brazilian soldier, was reported from Argentina in 1892 by Wernicke. Later, his assistant,

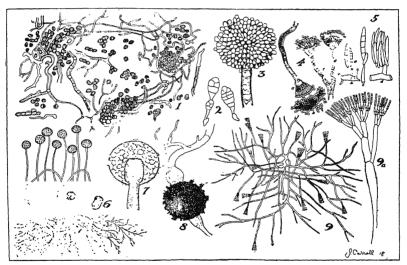


Fig. 43.—1, Tinca versicolor scrapings (Malassezia furfur). 2, Alternaria. 3, and 4. Aspergillus. 5, Fusarium. 6, 7, 8, Mucor. 9, 9a. Penicillium.

Posadas, made thorough studies of the parasite, including animal inoculation. Posadas regarded the parasite as a Coccidium, hence the name Coccidioides immitis. Clinically, this first reported case was like mycosis fungoides. Later study showed the parasite to be a fungus. In tissue we have more or less round cyst-like structures varying from 4 to  $80\mu$  in diameter, containing endospores and having a thick, doubly contoured capsule. These rounded elements never show budding in

tissues. Studies of cultures of the organism on artificial media proved the parasite a fungus. Creamy white colonies appear in 4 to 7 days on malt extract agar, later becoming cottony and brown, with abundance of chlamydospores (4 to  $7\mu$ ). The hyphae are about  $3\mu$  wide. MacNeal and Taylor have noted endospore formation in anaerobic cultures. Inoculation of monkeys, rabbits, guinea pigs and mice with cultures give the same cystlike structures, with endogenous spores, as noted in human tissues

By the end of 1935 more than 400 cases had been reported, with 80% from California, where the disease is reportable. Brumpt gives a species, C. histosporocellularis, as the

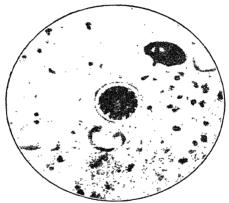


Fig. 44.—Coccidioides mmitis. Endogenous sporulation in center. (Courtesy of Commander H. E. Ragle, Mcdical Corps, U. S. Navy.)

cause in certain Brazilian cases, and Dodge notes the desirability of further comparison of the organisms as found in Argentina and the United States. Coccidioidal granuloma has also been reported from Mexico, and several states of the U. S., other than California. The disease is usually fatal within 3 or 4 years, and may start as a skin affection or first show as a pulmonary lesion. From either atrium of infection generalization may follow. It is this tendency to metastasis, through blood and lymph channels, that explains the seriousness of the disease. The differentiation from tuberculosis, syphilis and other fungus infections is in finding the non-budding cysts, with endospore formation. A wet preparation is entirely satisfactory for examination. It is a question whether the allergic skin reaction is reliable. Very little is known as to the source of infection, but the great resistance of the cultural spores, and the occurrence of laboratory infections, has suggested the inhalation of dried spores as the mode of inoculation rather than cutaneous entrance from dirt or plant material (thorns or prickles).

Rhinosporidiosis.— This polypoid disease is caused by a fungus, Rhinosporidium seeberi, which was first reported by Seeber in 1896 in Argentina. It was considered as a coccidial parasite, but the work of Ashworth (1922) proved it to be a fungus and not a protozoon. The organism is found within soft, very vascular, raspberry-like tumor masses, which are difficult to remove completely by reason of their friability and tendency to bleed profusely. The youngest forms measure  $6\mu$  and the largest ripe sporangia  $300\mu$  in diameter; they have a thick wall and contain thousands of nucleated spores. These polyps have been noted from nose, ears, conjunctivae and the mucosa of the penis. The infection has been reported from India, Cochin China, Uruguay and the United States as well as Argentina. Cultures and animal inoculations give negative results. The mode of transmission is unknown, but spores and ripe sporangia may be found in the nasal mucus.

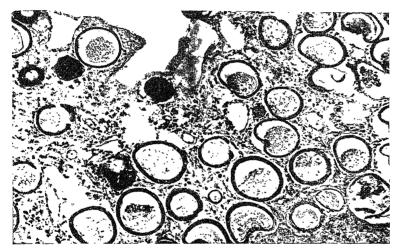


Fig. 45.—Rhinosporidium seeberi. Section from a nasal polyp. (U. S. Naval Medical School.)

Blastocystis hominis.—Ramsbottom considers this non-pathogenic fungus as belonging to the same grouping as Rhinosporidium. Blastocysts are frequently found in stool examinations and may be mistaken for amoebae or other protozoa. They are particularly abundant where the amoeba, Dientamoeba fragilis is found (symbiosis?). B. hominis is a parasite of the large intestine. It is a frequent contaminant of cultures made in examinations for amoebae. Brumpt states that this fungus is found in 50% of stool examinations. It varies from 3 to 15 $\mu$  in diameter and has a large central vacuole, which does not stain dark brown as does the glycogen mass of Iodamoeba. The protoplasm surrounding the vacuole varies in thickness in different sections of the ring and shows a varying number of nuclei. Budding forms may be seen.

Blastomycosis.—Gilchrist, in 1896, described the organism causing human blastomycetic dermatitis as encapsulated and budding in tissues,

with mycelial forms in cultures. Dodge places this yeast-like fungus in the genus Zymonema, Z. dermatitidis. Other species may be involved in blastomycosis. According to Vuillemin it was Cryptococcus gilchristi. In tissues we find spherical or ovoid cells singly or in groups, varying from 7 to  $20\mu$  in diameter. Budding forms are common and the thick, highly refractile membrane makes the cells appear as doubly contoured. In cultures, on solid media, we get about the third day a small, creamy,

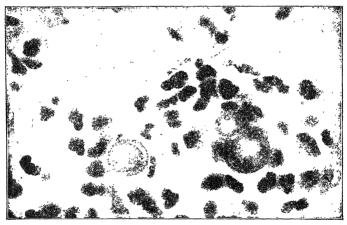


Fig. 46.—Z. dermatitidis (C. gilchristi). Doubly contoured organisms found in oidio-mycosis (blastomycosis.) (From Buschke after Hyde and Montgomery.)

prickled (coremia) colony with about  $3\mu$  hyphae. Chlamydospores  $(7 \text{ to } 8.5\mu)$  may be terminal or intercalary. The ascus in 8-spored. No pellicle on sugar broth. The guinea pig is susceptible to infection but the mouse is best, inoculated either intraperitoneally or intratesticularly. The infection is fully developed in the laboratory animal by the third week, showing yeast-like bodies in the tissue nodules. The disease may start as a skin lesion or this may be secondary to a primary pulmonary invasion. The skin lesions may resemble a verrucose tuberculid, a syphilitic gumma, or a sporotrichosis lesion. The exact diagnosis depends upon laboratory tests. In generalized blastomycosis the lung is involved in more than 90% of cases, and in skin blastomycosis it is secondarily invaded in about one-third of such cases. In primary pulmonary blastomycosis we have a bronchopneumonia from which the infection may generalize. There is less tendency to cavitation than in phthisis. The

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sputum is apt to be blood stained and may contain the fungus. Next to the lungs and skin the infection most frequently invades the kidneys, but the organism is not apt to be found in the urine unless we have invasion of the bladder or prostate. Next in order occur bone and central nervous system involvement.

Meningoencephalitis.—Dodge and Ayers isolated a fungus from the surface of the medulla in a case diagnosed as meningoencephalitis. The name of this parasite is  $Zymonema\ capsulatum$ . In the lesions only budding cells were found. In cultures thin-walled hyphac (3.5 to  $4\mu$ ) were noted. Chlamydospores developed from racquet mycelium.

#### THE MONILIASES

We have a large group of fungus infections which have been attributed to various yeast-like organisms, many of which show mycelial development in cultures. The best known of these moniliases is "thrush," in which greyish-white membranous patches form on the mucous membrane of the gums, tongue, buccal cavity and pharynx, chiefly affecting marasmic infants or feeble old people—it rarely attacks well nourished individuals. On examination of a fragment of such membrane we find yeast-like bodies which, when cultured, tend to show a yeast morphology on solid media and a mycelial one in liquids. In stab cultures of agar or gelatin we have yeast-like forms on the surface, but lower down in the stab (partial oxygen tension) we have an outgrowth of mycelial threads showing budding. The addition of extract of carrots to the standard media promotes mycelial formation.

Although the generally accepted name for this organism is Monilia albicans (the name Oidium albicans was given at an earlier date—1853), this "albicans" species has been assigned to many other genera, of which might be mentioned Saccharomyces, Mycoderma, Endomyces, Mycotorula and Dematium. Henrici has a table which gives a simple differentiation of the yeast-like organisms often referred to in moniliasis literature. In Endomyces we have both mycelium and budding cells, the mycelium forming asci by fusion of contiguous cells. Losing the power to form ascospores it becomes Monilia. Should Monilia lose the power to form mycelium it becomes Cryptococcus (asporogenous yeast). When Endomyces loses the power to form mycelium it becomes Saccharomyces, a true yeast, which never forms mycelium and reproduces by budding and the formation of ascospores (resulting from the conjugation of adjacent cells or by parthenogenesis). The false yeasts (Cryptococcus) reproduce by budding but do not form mycelium or ascospores. Castellani, with chief reliance on fermentation reactions, and little attention to morphology, presented an elaborate separation of species concerned in the various moniliases. It is astonishing how many species are found in the

literature based on a single cutaneous, pharyngeal or pulmonary condition from which yeast-like bodies were noted. Competent mycologists have in recent years stressed cultural studies with various media as is required for bacteria, along with determination of pathogenicity. Dodge has grouped the more important of these "monilia" parasites in the genera Castellania and Syringospora.

Clinical Types.—(I) Besides "thrush" and "perleche" (mycosis of the angle of the mouth of young children, with macerated whitish areas) we have moniliases of the perineal or inguinal regions of infants, particularly those poorly nourished. Probably many of these "monilias" are saprophytic and only develop in individuals of lowered resistance. Yeasts

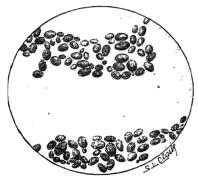


Fig. 47.—Yeast cells. Saccharomyces cerevisiae. (Coplin.)

are very common findings in the stools of normals, and these may find a suitable soil in cachectic children in regions adjacent to the anus. Some of the so called body eczemas and dysidroses of hands and feet are associated with "monilias." Many cases of "athletes foot," particularly where the toes are closely approximated, belong to the moniliases, although by far the greater number of such cases belong to the ringworm group.

In the Philippines, prior to arsphenamine days, I had examined for fungi many types

of syphilitic lesions (macular, papular and pustular). Of course the hot, moist skin of the tropics is generally absent in temperate climates, and the soil of an inflammatory lesion undoubtedly less suitable, but, in the tropics, with lesions definitely luetic, I was surprised at the proportion showing fungi in caustic potash mounts. To one unfamiliar with syphilitic lesions and history new causative fungi might have been reported. Exacting studies of the yeast-like bodies of sputum, faeces and inflammatory skin lesions would undoubtedly reduce the number of so-called "monilia" species which might properly be called pathogenic.

- (2) Many of the intertriginous ringworms of hands and feet are properly classed with the moniliases—in particular, cases of onychia and paronychia. It must be kept in mind that many skin lesions supposed to be due to fungi are really bacterial (staphylococcal, streptococcal and even due to the colon bacillus).
- (3) Cases resembling cutaneous blastomycoses and sporotrichoses have been found to show "monilia" forms of fungi.
- (4) Vaginal discharges may be due to so-called monilias, as well as to the better known trichomonad vaginitis.
- (5) In cases resembling chronic bronchitis or pulmonary tuberculosis a great number of "monilia" species have been reported.

(6) The best known of these "monilia" infections of the alimentary tract is "sprue." This disease is now recognized as a deficiency disease closely related to pernicious anaemia and responding to liver therapy.

Under the moniliases I shall consider only two genera, as given by Dodge—(1) Castellania and (2) Syringospora. These genera may be considered as belonging to the family Eremascaceae, order Endomycetales.

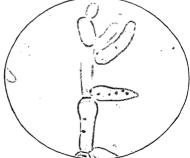


Fig. 48.—Endomyces vuillemini. Mycelial thread with four ripe chlamydospores; and conidia in the middle of the picture. (After Plaut.)

Castellania tropicalis.—Of the 53 species referred to Castellania and listed by Dodge I shall refer only to this species and C. pulmonalis, both of which were connected with pulmonary conditions incident to inspection of tea dust, inhaled by experts grading teas. Many of the species listed by Dodge are based on a single case and many of them fail to show any pathogenicity (saprophytes).

Syringospora albicans.—This is the classical fungus associated with thrush and referred to above.

Syringospora psilosis.—This fungus was regarded by Ashford as the cause of sprue. From the brief outline given it will be evident that this group of fungi should be restudied by trained mycologists, using every evidence from case material and animal inoculation, as well as cultural studies (morphological and physiological). It will be noted that in the table of fungi the genus Monilia is absent. Dodge states that species of Monilia are predominantly saprophytic. He gives only 2 species in his authoritative monograph, M. bonordeni, found on rotten wood, and M. kochii, found repeatedly in the sputum of a man. The latter seemed to Fig. 49.—Thrush fungus. have some connection with pyrosis.



Wassermann.)

Torulosis.—Freeman (1931) collected a number of cases of central nervous system involvement, which suggested neoplasm or encephalitis, but were associated with the presence of yeast-like organisms, Torula histolytica. Dodge has called this organism Cryptococcus histolyticus. Cryptococcus histolyticus reproduces only by budding, the yeast-like cells averaging 3 to  $4\mu$  in diameter. The whitish to yellowish colonies on glucose agar appear heaped up, smooth, pasty, shining and thick. They do not cause fermentation, pellicle formation or liquefaction of gelatin. The atrium of infection is probably pulmonary. The brain lesions show as tubercle-like formations and the lungs show the same appearance. Skin involvement is questionable. In a case reported by Harrison (1928), from a cystic blastomycosis of the cerebral grey matter, the organism liquefied gelatin after 26 days. Dodge has named it *C. meningitidis*.

The involvement of the cerebrum and meninges in torulosis suggests a brain tumor, particularly because of the eye symptoms, such as choked disc. A diagnosis may be possible by finding the yeast-like organisms in the cerebro-spinal fluid. Brumpt places the fungus of torulosis in the genus *Torulopsis* (*T. histolytica*).

The genera Cryptococcus and Malassezia are placed in the group Saccharomycetaceae Imperfectae (perfect stage unknown). As regards Malassezia, Dodge states that it is uncertain whether this genus belongs with the yeasts or the dermatophytes. As the very common skin lesion, pityriasis versicolor, is generally grouped with the dermatophytes, it would seem more convenient to deal with Malassezia under that grouping.

#### FUNGI IMPERFECTI (HYPHOMYCETES)

Under Saccardo's classification of the Fungi Imperfecti there are three main groups, Sphaeropsideae, Melanconieae and Hyphomycetes, but of these only the Hyphomycetes are involved in human pathology. Very confusing is the fact that in this class of fungi various workers assigned to different stages of the same fungus different generic and specific names. The difficulty is that with the Hyphomycetes we do not know exactly the complete life cycle. Dodge states "Hence, the Fungi Imperfecti are conceived as a temporary dumping ground until the gaps in our knowledge have been filled."

Pityriasis Versicolor (Tinea Versicolor).—This exceedingly common skin affection is characterized by dirty yellowish-brown spots about covered parts of the body, especially under the clavicles. These spots are sometimes referred to as "liver spots." The plaques are not elevated and do not show inflammation. A vigorous sweep of the thumb nail across the patch (a sterile instrument preferable) does not bring blood (Signe du coup d'ongle). The scrapings provide material showing an abundance of mycelium and spores of Malassezia furfur. The hyphae are from 2 to  $3\mu$  in diameter and the spores from 3 to  $8\mu$ . The spores are very refractile and may show budding.

On glycerin agar tiny white drops appear after a week. On Sabouraud's medium the primary growth is exceedingly slow (months to produce a colony the size of a rice grain).

Erythrasma.—This is sometimes confused with pityriasis but is a distinct skin affection which is caused by  $Actinomyces\ minutissimus\ (Microsporon\ minutissimum).$  The mycelium averages  $\iota\mu$  in diameter, often fragmented into bacillary arthrospores. Cultures are usually negative. Erythrasma spots are dark red to brownish (usually in the groin region or axilla). It is often confused with trichophytosis or lichen, as well as with tinea versicolor.

## THE RINGWORM SKIN AFFECTIONS

These dermatomycoses may vary greatly clinically, even as regards ring formation, but are grouped together because they tend to involve only the epidermis. The chief

parasitic fungi invading the superficial skin layers belong to the family Trichophytoneae, and Dodge considers these dermatophytes as, probably, imperfect stages of Gymnoascaceae. Sabouraud's classification of these fungi is based on both clinical and mycological grounds, and has been the guide of most dermatologists all over the world for the past forty years, notwithstanding numerous valuable studies and classifications made by Castellani and Chalmers (1919), Vuillemin (1925), Langeron and Milochevitch (1930) and others. Dodge has preferred to follow Sabouraud (1910, 1928, 1929, 1932) as his guide, so that the classification adopted from Dodge, in this little book, is essentially that of Sabouraud.

The ringworms involving both scalp and glabrous skin are the endothrix group, the ectothrix group, the microsporum group and favus.

The Endothrix Group.—It is usual to divide this group into an endothrix and a neo-endothrix group. In the former, the hyphae are confined to the interior of the hair, while in the latter, in addition to hyphae in the interior, we have a few hyphae growing along the outside of the hair, Arthur Whitfield notes that early in the invasion of the hair we find the fungus in the root sheath as well as within the hair, but the external ones soon die out, leaving only the hair invasion. In the so-called neo-endothrix infections, the invasion of the root sheath continues for a longer period.

In the Malmstenia subtype of ringworm of the scalp (Trichophyton tonsurans), we have many normal hairs in the area of alopecia, and the diseased hairs project from 2 to 4 mm. above the scalp surface. The scales covering the affected spot, if gently removed, show flattened, diseased hairs lying beneath them, often assuming bizarre shapes. This infection rarely itches. It is a disease of young children and disappears at puberty—it has been suggested that gonadal activity explains the age exemption. It is very contagious. The quadrangular cells of the hyphae (often called spores) are 4 to  $5\mu$  long. It gives a powdery, crateriform culture on Sabouraud's medium. Guinea pigs can be infected. This ringworm also occurs on the face, neck and hands of children showing the infection as red areas with little vesicles or papules. It has been reported occasionally from cases of otomycosis and onychomycosis.

The Sabourandia subtype is caused by Trichophyton sabourandi (T. acuminatum) and is quite common in western Europe but of less frequency than the Malmstenia Tinea tonsurans. The hairs break off close to the scalp scalp patches, and show as black dots on the scalp surface. It may cause a trichophytosis of the hands and, secondarily, of the nails. It may invade the beard. The aleurospores are from 5 to  $7\mu$  in diameter and very fragile. Cultures are rather cupola-like.

The Ectothrix Group.—In this group mycelium is found both within the hair shaft and in the root sheath surrounding the hair. The designation "endo-ectothrix" has also been used for the ectothrix group. Sabouraud gives a megalospore and a microides subtype. The chief species of the large spore group is Megatrichophyton roseum, which is rather common in the north of England, where its spread seems to be through the

barber shops, rather than from contact with animals. It has spread rather widely since the war, being reported from France, Germany, and in the U. S. (Philadelphia).

The lesions are of the skin or of hair follicles, but chiefly of the beard, and are rather dry, showing no tendency to suppuration.

In the "microides" group the chief offender is Ectotrichophyton mentagrophytes (Trichophyton gypseum var. asteroides). The lesions of the scalp or beard (sycosis vulgaris is a beard folliculitis caused by pyogenic cocci) are attended with marked inflammation, going on to suppuration of the follicles containing the dead hairs. This fungus is probably the most common cause of kerion. On the glabrous skin we have patches riddled with openings from which pus oozes on pressure. These patches are usually on the backs of the hands or forearms, and occur most frequently in those who work with horses. When on the face or scalp, the affected hairs can easily and painlessly be extracted from the root sheath; they are, however, quite brittle and may break off. Epilation of the infected hair shafts is indicated. On mounting in caustic potash solution we find strings of spores  $(5\mu)$  on the hairs extracted from the periphery of the lesion, or in the pus. The sheath at the base has spores varying greatly in size  $(2 \text{ to } 11\mu)$ . The "microides" spores of the sheath may be confused with the small spore sheath of Microsporum audouini, but the former are arranged in chains while the latter show masses of polyhedric spores, about  $3\mu$  in diameter.

The Microsporum Group.—These small spored ringworms of the scalp are the most common ones in France and the United States. It is customary to recognize a human type which is important only for the scalp, as the lesions which may appear on the glabrous skin of the neck are rather insignificant and come from scalp transfer. It does not invade the beard. The name of this fungus is Microsporum audouini. The areas of alopecia of this human type show greyish scales, covering an area with definitely outlined borders and covered with diseased hairs, broken off about 3 to 4 mm. from the scalp surface. The area never shows any normal hairs. On epilating one of these greyish stumps of hair we note a whitish collar of polyhedric spores (2 to  $3\mu$ ), which never penetrate the shaft of the hair. The spores of other common types of scalp ringworm tend to be larger (5 to  $7\mu$ ). This infection attacks young children almost exclusively, and Brumpt advises us to think of an animal origin when we find one of these small-spored ringworms in an adult. The human type rarely causes even superficial pustulation. Of course, this may occur from bacterial infection in scratching by the child.

The animal species of Microsporum (Sabouraudites) which have been chiefly studied are those belonging to the horse, the dog and the cat. That of the dog, M. canis (M. lanosum), produces both tinea tonsurans and circinate herpes in both children and adults. The lesions are generally scaly and dry. The cultures grow more rapidly than those of the human type and they show more abundant fuseaux. The cat species (M. felineum) seems to prefer the glabrous skin, producing dry erythematous lesions, which may go on to pustulation. Some kerions are due to the cat ringworm. It grows very rapidly in cultures. It is common in England, but less so in the United States.

Favus.—This disease usually affects the scalp hairs, although invading the glabrous skin and nails. It is characterized by golden-yellow, cup-

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shaped crusts (scutula), which form about the hair follicle orifice. The scutulum is made up of tangled mycelium surrounding the hair with vertical mycelium externally and, underneath, a pus cell layer. The scutula may remain isolated, or form dirty crusts on coalescence. If this impetiginous crust is removed the yellow color appears. The odor of old favus lesions is that of a mouse nest. The infection is not particularly contagious. The causative fungus is Achorion schoenleini (Grubyella).

The affected hairs are lusterless and greyish, and while more fragile than a normal hair can be epilated entire. Scarring may result. The favic hair contains few hyphae while the trichophyton hairs are generally filled with spores, which accounts for the greater fragility of the latter. The favic hair is filled with air bubbles. The septate hvphal chains vary greatly in diameter (2 to  $5\mu$ ). Cultures on Sabouraud's medium are like yellow beeswax and resemble cerebral convolutions. The growth is much slower than that of Trichophyton, and on microscopic examination there are fewer fuseaux. On the glabrous skin we may have scutula, but more commonly scaly patches. The involvement of the nails is rare and always follows lesions elsewhere (scratching). The toe nails are very rarely attacked. In the absence of a prior human case one must suspect infection from an animal source, particularly from mice, and caused by Achorion muris. This is a serious favic disease in mice, attacking the head and leading to blindness. In the human infection the lesions are mostly on the body (like herpes circinatus). It is reported as common in Germany, less so in England and France. The classical favus is common in China and central Asia, but rare in Japan, England and America. In the U. S. cases are more common in immigrants from eastern Europe.

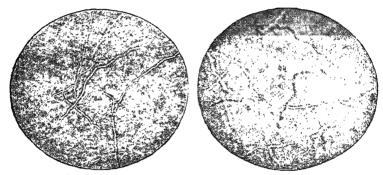
Eczema Marginatum and Ringworm of the Hands and Feet.—The above epidermophytoses are characterized by invasion of the horny layers of the epidermis alone. The ringworms of the hands and feet are generally caused by Epidermophyton interdigitale, and Hebra's eczema marginatum by E. floccosum (E. cruris?).

We may separate the ringworms of the genera *Epidermophyton* and *Endodermophyton* from those just considered by the fact that they do not attack the hairs. Even in tinea imbricata, when the *Endodermophyton* spreads to the scalp, it always respects the hairs. In *Epidermophyton* infections the fungus is found, characteristically, in the stratum corneum of the epidermis, while with *Endodermophyton* the typical location is between the stratum corneum and the stratum lucidum of the Malpighian layer of the epidermis. These fungi never invade the corium.

The *Epidermo phyton* dermatomycoses are wide spread in both temperate and tropical climates, while the *Endodermo phyton* ones are strikingly restricted to the tropics. Manson's description of the fungus of tinea imbricata (Tokelau ringworm), from his studies of the morphology of the organism as seen in the scales, and his accurate report of the clinical course of the infection following human inoculation, is a classic.

The two genera are best differentiated culturally. In cultures of *Epidermophyton floccosum* we have a profusion of club shaped clostereospores (fuseaux), often in groups of 5 to 7, and divided by about 4 septa. With *Endodermophyton* we do not have fuseaux.

Epidermophyton infections.—Hebra described his eczema marginatum in 1860, and since that time very little has been added. The lesions are red and circinate with a distinct border, and usually start on the inner surfaces of the thighs, spreading to the groin regions. The infection may extend to the axillary or submammary regions, or to the feet and hands. In temperate climates it is very common in schools, barracks and gymnasiums, where it is probably spread by towels, clothing or the floors of shower baths. In the tropics the infection is called "dhobie itch," and this extremely common skin affection differs from the European type only by more angry looking and more swollen patches, which itch intolerably, and give rise to various eczematoid lesions as the result of scratching. The lesions of the hands and feet are so well known under the



Low Power
Fig. 50.—Epidermophyton floccosum, from skin scrapings. (Low and high power.)

designation "athletes' foot," that it seems unnecessary to describe these dysidrosiform and intertriginous dermatomycoses, which usually start in the interdigital spaces, thence spreading to the palms and soles. Dodge gives *E. interdigitale* as the principal cause of epidermophytosis of hands and feet and *E. floccosum* as that for eczema marginatum. The growth of the interdigital fungus is more rapid than that of the one affecting the inguinal and perineal regions. In the body ringworms we should get scrapings from the borders of the lesions, and in the interdigital ones, preferably, from the small greyishblue, sago grain-like vesicles, if present. The material is cultured and examined microscopically in caustic potash mounts. These ringworms do not invade the dorsal surfaces of the hands and feet.

Endodermophyton infection.—The type species is E. concentricum, the cause of tinea imbricata. In this tropical ringworm we have rosette-like lesions of several concentric circles of shingle-like, papery scales, which are attached peripherally and are free towards the center. As such circles extend they meet other circles and give rise to the watered silk appearance of the affected body surfaces. There is an entire absence of inflammation of the lesions, thus distinguishing them from dhobie itch. Itching is severe and secondary infection may occur from scratching. E. indicum has been reported as the

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cause of tinea imbricata in Malayia. Some authorities consider *E. concentricum* as less common in tinea imbricata, as reported from islands of the Pacific, than other endodermophytons.

#### THE ASPERGILLOSES

Various affections of the skin (including mycetomas), ear, and lungs have been reported as aspergilloses. The Aspergillaceae family, to which belong the very common saprophytic fungi of the genera Aspergillus and Penicillium, is responsible for many of the contaminants one finds in the study of plates in the bacteriological laboratory. These colonies, together with those of the yeasts, mucors and spore bearing bacteria. should be familiar to every laboratory worker. Just as the various species of the Aspergillaceae contaminate a plate, so do they at times find a suitable soil in skin, ear or lung lesions, and many of the mycological authorities question the importance of these fungi as primary excitants of disease. In this family we shall consider the genera Aspergillus, Penicillium, Scopulariopsis and Allescheria. In the study of cultures and microscopical preparations of fungi, it is assumed that the worker is familiar with the more or less round vesicle of Aspergillus, with its phialides (sterigmata) and the chains of conidia branching off from them. In Penicillium these conidial chains extend from a conidiophore (sporophore), which does not spring from a vesicle. It somewhat resembles the skeleton of a hand, including the carpal and metacarpal bones, as well as the phalanges (represented by the chain of spores). Material for microscopical preparations is abundantly at hand in the patches of moulds on decaying fruits or vegetables, as well as in contaminated plates. The very common blue-green mould is a Penicillium-P. crustaccum. Another species, P. brevicaule, when grown in material containing arsenic, produces a strong odor of garlic, and is the basis of a very delicate test for arsenic-1 in 1,000,000. The very black moulds will in many instances show the fruiting bodies of Aspergillus niger. A. oryzae saccharifies the rice starch (diastase) and, in symbiosis with a yeast, produces the 15 per cent alcoholic sake of the Orient.

Other fungi of this family are of great commercial importance. In material from sputum, pus or skin scrapings, we get a morphology which does not resemble the fruiting bodies obtained by culturing this material in media containing fermentable carbohydrate, hence the study of pathological material should always include cultures. If any species in this group is capable of producing disease primarily, it must be Aspergillus fumigalus. This fungus appears to be responsible for an epizootic in pigeons, and pigeon-feeders have furnished cases of aspergillosis. This infection may be found in parrots and should be considered in a human bronchopneumonia, as well as psittacosis. This pulmonary infection resembles phthisis but the patient does not seem to show illness equal to tuberculosis. Haemorrhages are frequent and, at other times, the sputum tends to be blood-streaked. Cases of aspergillosis have been reported from nasal and corneal lesions; in the latter case the injury has been from scratches by plant material. A. unguis has been reported as the cause of a mycosis of the great toe.

Mycetomas.—While mycetomas are preponderantly connected with species of Actinomyces, yet cases have been reported where the findings were those of Aspergillus and Penicillium. A white grain mycetoma, Allescheria boydii, was reported from a single case in the United States. While the evidence for pathogenic action of Penicillium is slight, this cannot equally be said for Scopulariopsis, which morphologically greatly resembles Penicillium. Species of this fungus have been reported from cases of

onychomycosis. (S. brevicaulis and other species.) S. koningi was reported from a gummatous lesion of the hand and, in several instances, from trench foot.

#### CARATÉ OR PINTA

This rather widespread dermatomycosis of Mexico, Central America and Columbia shows variously colored spots on the face, backs of hands and body. Fox (1935) notes its prevalence in southern Mexico, the blue and white pintas predominating. While some have reported species of Aspergillus and Penicillium as obtained from scrapings, it would appear that the most common fungus is Dematium wernecki. On carrot this fungus produces a mycelium of short cells with simple blastospores. Brumpt gives a list of 27 species of fungi reported by various authors. Recent views regard the fungi obtained from scrapings of the lesions as common saprophytes. The old idea that differently colored pintas were caused by specific fungi is no longer tenable. In fact, the different colors seem to be connected with varying degrees of atrophy of the derma, which finally results in white spots, similar to vitiligo. Furthermore, varying colors seem to be related to racial or environmental factors. Whatever the true pathology of this chronic skin disease may be, the frequency of positive Wassermann reactions is striking, Menk giving 75% of positives in a series of cases. However, epidemiological evidence does not support the view of a syphilitic origin.

#### THE MADUROMYCOSES

Van Dyke Carter established the fungus nature of Madura foot, a common affection in the Madras Presidency of India. It is less frequent in other part of India. It is found in northern Africa and has been reported from Italy and Greece, and even from the American continent. Carter applied the designation "mycetoma" to the disease, and Chalmers and Archibald divided mycetomas into two groups: (1) Maduromycoses, with granules containing large segmented mycelium, with well defined walls, and often chlamydospores; and (2) Actinomycoses, with granules composed of very fine, non-segmented filaments, with ill-defined walls and no chlamydospores. They also recognized a paramycetoma, in which the fungus did not produce granular aggregations. The maduromycoses have been separated on the basis of the color of the granules, black, white, yellow and red. Usually only one kind of fungus is found in a single case.

The most common fungus of the black grain maduromycosis is *Madurella myceioni*. As a rule the mycosis is limited to the foot, but may involve the hand. It is supposed

into the tissues of the foot or hand by puncture of a thorn gus develops in granulomatous areas, from which lead

discharging a viscid, seropurulent fluid containing the black grains (sclerotia). may be only nodules, or the entire foot may be swollen and distorted. Visceral tases never occur. It is the burdensome weight of the foot, rather than the pain, is complained of. The mycelium of Madurella is septate and much

(usually less than Tu in diameter while the hyp

may

reach a diameter up to of white caused by species of ni (India).

#### ACTINOMYCOSIS

In discussing Bergey's classification (Chapter 1), it was noted that he grouped the genus Actinomyces with Mycobacterium (tubercle bacillus) and Corynebacterium (diphtheria bacillus), genera definitely belonging to the Schizomycetes or true bacteria. The family of Actinomyceteae is so widely distributed in nature (in the soil, on plants and in the faeces of man and animal), and so important in biological processes, that its careful study has been the province of biologists, rather than those interested in human or veterinary medicine, and there seems definite agreement among them to assign this group of organisms to the domain of mycology.

Characteristics.—Actinomyces resembles bacteria in many respects, of which the following may be noted: (1) The diameter of the mycelial threads rarely exceeds  $1\mu$  (usually 0.5 to 0.8 $\mu$ ). (2) The sparsely and irregularly septate filaments of Actinomyces tend to break up into bacillary or coccoid forms. This fragmentation has been interpreted as arthrospore formation, and in some species chains of spores are noted. The sporogenous hyphae frequently show a coiling of several turns (rarely exceeding 12 spirals), which may be to the right or left. The spirals uncoil after the maturation of the spores. Variations in spirals are of importance in the separation of species. All species are non-motile and Gram-positive. Some species, when grown on fat-containing media, may show acid-fast staining.

It must be kept in mind that many of the attempts at culturing the acid-fast leprosy bacillus have resulted in streptothrix (Actinomyces) growth, and Deycke's nastin (a much vaunted cure for leprosy) was an ethereal extract of such a streptothrix growth obtained by culturing leprous nodules.

In common with other mould contaminants of bacterial plate cultures, we frequently find leathery, very adherent colonies of Actinomyccs, which may be chalky or show various colors—yellow, red, black or green. When transferred, such colonies give off a musty, earthy odor. In medical mycology a separation on the basis of oxygen requirements has been used, one group requiring anaerobic conditions for growth and the other growing under aerobic environment. The pathogenic species attaching to actinomycosis in man, or "lumpy jaw" in cattle (the Isreal type), belongs to the anaerobic group. The requirements are rather those of partial oxygen tension than complete anaerobiosis. J. H. Wright stressed the necessity for choosing as material for culture that which contained filamentous organisms (observed by pressing between two slides), and then for inoculating glucose agar stabs.

Growth first appears one or two cm. below the surface. Bacterial contaminants are frequently responsible for failure. Growth is first noted as a small white speck, after three days, at 37°C. It would seem probable that the parasitic species producing

actinomycosis belong to the anaerobic group, while the aerobic division is largely saprophytic. The Actinomyces genera of mycetoma seem, however, to be mostly aerobic. Bostroem has claimed that he has isolated an aerobic fungus from actinomycosis but his work, generally, has not been confirmed. Wright called the aerobic group Nocardia and the anaerobic one Actinomyces. Other generic names are Streptothrix, Oospora and Discomyces.

Clubs and granules.—In the pus from lesions we have the sulphur granules in actinomycosis, and the black (gunpowder) ones in Madura foot. Mycetomas caused by other fungi may show granules of other colors. The granules of  $A.\ bovis$  rarely exceed 150 $\mu$  in diameter and are

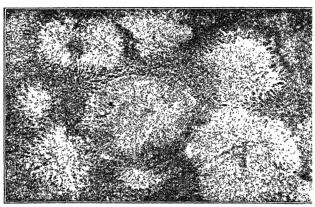


FIG. 51.—Actinomyces granule crushed beneath a cover glass, showing radial striations in the hyaline masses. Preparation not stained; low magnifying power. (McFarland after Wright and Brown.)

of a yellowish white color. The granules of A. madurae are of an ochroid color and cause Carter's white mycetoma. The fungus of the black grain variety of mycetoma is Madurella mycetomi. These granules (when small and crushed between slides) show a central filamentous mass with bulbous structures set peripherally (usually called "clubs"). Dodge considers these granules as bulbils (small sclerotia). Clubs are rarely seen in cultures. It is from the peripherally placed bulbous extremities that we get the name "ray fungus."

Clinical manifestations.—There are four main types of actinomycosis in man—(r) Cutaneous, where an epidermal lesion invades the corium and subcutaneous tissues producing a nodule which breaks down and discharges fish-roe granules. (2) Buccal. In cattle the invasion of the jaw

has given this actinomycosis the name "lumpy jaw." The process extends to the adjacent soft parts. The tongue is involved in almost one-third of these cervico-facial types. (3) Usually secondarily to buccal infections we have a pulmonary type, usually fatal, while the purely buccal ones, which can drain freely, generally recover. (4) The intestinal type, usually in the region of the caecum and appendix (diagnosis commonly appendicitis). The actinmycotic lesions are generally free from pain. Extension is by continuity—rarely by blood vessels or lymphatic channels. When enlargement of tributary glands occurs it is indicative of

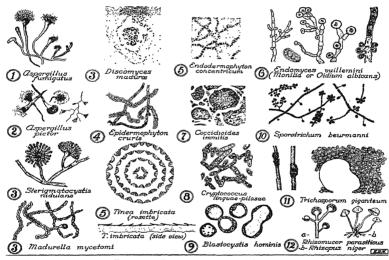


Fig. 52.—Important tropical fungi.

Actinobacillus infection. The diagnosis of actinomycoses depends chiefly upon laboratory examinations.

Epidemiology.—Heating to 60°C. is always fatal to these organisms, so there is no question of a resisting spore, as for anthrax. For many years the opinion was held that infection took place by inoculation of some wound produced by plant material (as a blade of grass or a cereal grain). In view of the fact that A. bovis is a rather delicate, anaerobic organism and grows only at body temperature, this idea is untenable. There is much to indicate that actinomycotic filaments may have as habitat the carious material in defective teeth or in tonsillar crypts. Naeslund (1925–1926) has isolated anaerobic strains from such material, which may be identical with A. bovis. He also considers tartar formation on the teeth as a possible source of infection.

Laboratory diagnosis.—The fungus is to be found in the granules, and it is essential that culture or microscopical material be obtained from them. The purulent discharge may be poured in a thin film in a Petri dish and search made for these minute granules. They are best fished up with a capillary pipette and examined unstained, with diminished light, for the clubs of the ray fungus. In tissue sections Gram's stain is very satisfactory, although they show beautifully in ordinary haematoxylin-cosin stains. The method of culturing used by Wright is noted above. Very satisfactory is the use of the capillary portion of a rubber bulb pipette. The growth in this narrow casing can be satisfactorily observed under the microscope. Animal inoculation has not been satisfactory, as Homer Wright was not able to satisfy himself of actual multiplication in laboratory animals.

More important species.—Dodge gives the characteristics and synonyms of 108 species of Actinomyces with a differentiating key.

- A. bovis.—This organism is generally considered the cause of lumpy jaw in cattle, and is present in the sulphur granules which are so characteristic of this infection. The fungus which is present in human actinomycosis seems to have the same morphological and cultural characteristics as that of cattle. As noted above, it requires partial O tension and body temperature for growth. The problem of etiology is complicated by the frequent association with actinomycosis of two bacterial organisms, B. actinomycetem comitans and the Actinobacillus. It is even maintained that many of the pathological changes present may be due to these companion organisms.
- A. madurae.—This organism is the cause of white mycetoma (Carter or Vincent). The granules are white to yellow and the hyphae from 1 to 1.5 $\mu$  in diameter. Authorities disagree as to cultural requirements. According to R. St. John-Brooks, it is a strict aerobe growing best at 37°C., but having a wide temperature range (20 to 40°C.). Dodge notes that it is a facultative anaerobe, but grows best anaerobically.
- A. asteroides.—This is a frequent cause of white grain mycetomas in various parts of the world. Some authorities give its cultural requirements as aeroanaerobic—others as aerobic, and still others as anaerobic. This fungus was first reported by Eppinger from a brain abscess and, apparently, the same organism has since been reported from mycetomas in the Philippines, Brazil, Argentine and Europe, as well as Asia and Africa. Brumpt gives it as Gram-positive and acid-resistant.
- A. gypsoides.—This organism was isolated by Henrici and Gardner from the sputum of a case of pulmonary mycosis. They noted its growth as more rapid than that of the tubercle bacillus and that it was acid-fast. For its recovery from sputum they recommended guinea-pig inoculation. Wright could not assure himself of multiplication of Actinomyces in guinea-pigs.
- A. keratolyticus.—This Actinomyces is given as the cause of cracked heels in India (keratolysis plantare sulcatum). It has also been found in interdigital erosions. It is said to be due to walking barefooted on damp soil covered with manure.
- A. minutissimus.—This fungus has been reported as the cause of erythrasma (see p. 220).
- A. thuillieri.—This fungus was studied by Pasteur and Rosenbach (1883–1884). It was the first fungus with which proof of immunity was obtained. It seems to live saprophytically on fish. It causes a disease of pigs known as swine erysipelas, which may be communicated to butchers, veterinarians or others working with this pig infection. In Europe this disease of pigs is serious economically, but it is rather rare in the U.S. In Germany it is called rollauf, and in France rougel. Besides the hog, the dis-

ease may be communicated by other animals, especially crabs. Most cases in the U. S. have come from handling crabs or fish taken in the Chesapeake Bay region. The human disease, <code>crysipeloid</code>, follows local infection of a wound of the skin. In a day or two a swollen, deep red area, with a bright red margin appears. Although the usual type of infection is cutaneous, rarely it may produce lymphangitis. Glandular enlargement may occur. Rarely, the infection may become generalized. The organism resembles the diphtheroids, but is now classed with the Actinomyceteae. Another name is <code>Erysi-</code>

pelothrix rhusiopathiae and it was formerly called B. erysipelatis suis. It is easily cultured and on bile media shows filamentous forms, whereas in broth we get only bacillary forms. A pH of 7.6 favors growth. The infection seems to confer a lasting immunity. An antiserum has been produced which may be injected into the region of the lesion or intramuscularly.

#### Sporotrichosis

This gummatous or ulcerative mycosis, which usually follows the course of the lymphatics of forearm and arm, generally starts from a thorn prick of hand or phalanges. The causative fungi of the genus *Sporotrichum* are widely distributed as saphrophytes. The family of Sporotricheae is characterized by branched, septate mycelium. The spores, which are generally single and never in chains, project from the hyphae on short sterigmata, or are sessile.

From the standpoint of reported cases, France and the U. S. are the chief countries showing infection, but this may be connected with more frequent search for the fungus—the isolation of the fungus from a sporotrichosis lesion having been first reported by Schenck in 1898 (J. H. H. Bulletin), and later on recognized as a clinical entity by Beurmann and Raymond (1903). Dodge assigns S. schencki as

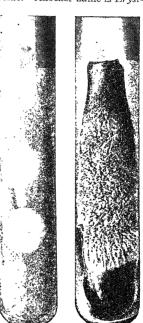


Fig. 53.—Sporotrichum schencki. Cultures on the glucose peptone agar of Sabouraud. (After Gongerot.)

the name of the parasite, and considers S. Beurmanni a variety. The hyphae are about  $2\mu$  in diameter. A difference between the two organisms has been noted in the frequent appearance of chlamydospores in the Beurmann fungus, and the rarity of such resistant spores in the Schenck fungus. In the pus or tissues of sporotrichosis lesions one never finds mycelium, but only cigar shaped, yeast-like bodies, phagocytized in monocytes. For diagnosis one should always culture the pus or scrapings (preferably from an unopened lesion). The colony first appears about the second day, and a microscopic preparation shows the narrow mycelium with spores (3 to  $5\mu$ ), usually in groups at the ends of the

hyphae. Optimum temperature is from 30° to 38°C. Sporotrichosis is usually diagnosed as syphilis or tuberculosis, but development of the lesions is more rapid. There is also less effect on the general health, as well as a lack of glandular involvement. Cases of pulmonary sporotrichosis have been rarely reported, and also generalized infection. The lesions are usually confined to the deep parts of the skin or mucous membranes.

Laboratory diagnosis.—Widal and Abrami have proposed an agglutination test, and others, skin tests, but the culturing technique is so simple and satisfactory that it is given preference. An examination of the pus, microscopically, rarely reveals the cigar-shaped rods. A caustic potash mount is satisfactory if the large bacillary forms are present, but it is on the culture we depend for diagnosis. Sporotrichosis is often associated with tuberculous lesions.

Sporotricheae in Tinea albigena and Mycetoma.—A skin infection of hands and soles, later extending to arms and legs, seems quite prevalent in the East Indies. The fungus is Aleurisma albiciscans. A black grain mycetoma has been reported from the Soudan (Trichosporium khartoumensis).

#### METHODS OF EXAMINATION

Similar methods apply in the study of pathogenic fungi and bacteria, the objective being the separation of species or varieties either by isolation of pure cultures or the observation of pathological effects in animals.

As a rule the growth and appearance of colonies of fungi are more easily observed than are those of bacteria, and the morphology more characteristic microscopically. In the study of culture plates we necessarily become familiar with a great number of mould contaminants which we readily recognize at a glance. Castellani has attached great importance to fermentation reactions, but it is generally recognized by bacteriologists that many factors make such methods questionable in species determination. In scrapings from the skin, pus from discharges, sputum, faeces, vaginal discharges or various exudates the standard practice is to mount in a 5 to 10 per cent solution of caustic potash. For dried scales and hairs, strengths of 20 to 30 per cent are used. Artifacts resembling spores or mycelium are confusing in caustic potash mounts. One should familiarize himself with such artifacts in normal skin preparations. For pus, sputum, empyema fluid, or various exudates and discharges, mounting in Lugol's solution is desirable.

Tribondeau's method is to treat the scales with ether, then with alcohol, and finally with water. Next put the sediment (it is convenient to use a centrifuge) in a drop of caustic soda solution. Cover with a cover glass, and after the preparation has stood about an hour run glycerin under the cover glass.

A very satisfactory method is to scrape the scales with a small scalpel, and smear out the material so obtained in a loopful of white of egg or blood serum on a glass slide. By scraping vigorously the serum may be obtained from the patient. After the smear has dried, treat it with alcohol and ether to get rid of the fat. It may then be stained with Wright's stain or by Gram's method. The ordinary Gram method may be used, or the decolorizing may be done with aniline oil, observing the decolorization under the low power of the microscope.

Yeasts are best examined in hanging drop on a plain slide with vaselined ring.

An excellent way to examine many common moulds is to seize some of the projecting sporangia from the surface of a plate with forceps and mount in liquid petrolatum. I have found that moulds in scales from skin or from infections of various mites or insects will show a growth in this medium when mounted on a slide and covered with a cover glass. The mycelium grows out from the body of the arthropod.

For the microscopic study of moulds it is well to clear and mount them in lactophenol (carbolic acid, 20 cc.; lactic acid, 20 cc.; glycerin, 40 cc.; water, 20 cc.). This may be tinted with some dye, as aniline blue. The above is Amann's formula.

Dodge recommends a r per cent aqueous solution of cotton blue, a drop of which is placed on a slide and this is inoculated with the fungus material. A coverslip is applied and then plain lactophenol is drawn under the coverslip. A piece of filter paper at one side of the mount absorbs the aqueous solution and draws in the lactophenol drop on the other side of the square coverslip. The supravital blood staining method is of value in the study of pus, sputum or exudates containing fungi.

#### CULTIVATION OF FUNGI

Moulds grow well on media with an acid reaction, so that by adjusting the reaction to +2, or even higher, we permit the growth of the fungi, but inhibit bacterial development.

Glycerin agar, bread paste, or potato media are all suitable, but the standard media are those of Sabouraud:

Conservation medium (for preserving stock cultures).

	Gm.
Peptone	30.0
Agar (shred)	15.0
Tap water	1000.0

Differentiation media (two of these are used—a maltose medium and a glucose medium).

	Gm.
Maltose	40.0
Peptone	
Agar (shred)	15.0
Tap water	1000.0

The glucose medium is made by substituting glucose for maltose in the above formula.

In each case the ingredients are added to the water and all placed in a cold autoclave and the pressure allowed to rise in both outer and inner jacket simultaneously until it has reached 15 pounds. The autoclave is then shut off and allowed to cool down slowly. When autoclave has cooled, the media is filtered through cotton, tubed, and then sterilized the same as above. Upon removal from the autoclave, the tubes are slanted and allowed to cool in this slanting position.

The media is not titrated or the hydrogen-ion concentration adjusted in any way, Sabouraud claiming that the addition of either acid or alkali spoils the media.

Some mycologists claim that the Sabouraud medium contains too much sugar and that this interferes with growth and morphology. The monosaccharides seem to be more objectionable than the polysaccharides. Besides potato slants, such solid media may be made from carrots in particular, but any fruit or vegetable may be similarly prepared. Some use slices of raw fruits or vegetables, deposited in moist-chamber Petri dishes. Asepsis must be rigidly observed in making the slices of raw fruits or vegetables. Mushrooms have been recommended for the study of the dermatophytes. Horse manure is often used for the study of mycelial (not yeast) growth. It should be as fresh as possible and thoroughly sterilized.

Carrot agar is used by many: Carrot, 500; agar, 20; peptone 10, and water to make a litre. Mushroom agar may be tried. Brain media have a distinct value. The Digestive Ferments Company sell prepared media, which have an advantage in standardization (comparison of culture results from different laboratories).

Before inoculating media with moulds, some recommend placing the material in 60% alcohol for one or two hours to kill the bacteria. The moulds withstand such treatment.

In cultivating moulds, small Erlenmeyer flasks, containing about ¼ in. of media on the bottom, will be found suitable for the development of the colonies. In order to isolate the mould we may take the hair or scales on a sterile slide and cut them into small fragments with a sterile knife. Then moisten a platinum loop from the surface of an agar slant, touch a fragment with the loop, and when it adheres transfer it to the agar slant. Make four or five inoculations on the surface, and from suitable growth, after four to seven days, inoculate the medium in the Erlenmeyer flask.

Plaut recommends: Place the mould material between two sterile glass slides. Seal one edge with wax and place the preparation in a moist chamber for four to seven days. From the fungus growth developing inoculate the medium in the Erlenmeyer flask. A Petri dish containing several layers of thoroughly moistened filter paper in top and bottom makes a satisfactory moist chamber.

Hanging block cultures.—One of the best methods of identification of moulds is to fill the concavity of a hollow slide, which has been flamed for sterilization, with melted Sabouraud agar or other media suitable for moulds. The surface is then inoculated with material from the colony to be studied and a flamed (sterile) cover glass applied. In a similar method, which was devised for the study of bacterial cultures, IIill used I cm. squares of agar, cut out from sheets made by pouring melted agar into Petri dishes. Another method is to let a large drop of melted agar spread over a sterile cover glass and then inoculate the film of medium and adjust over a concave slide. These methods are excellent for bringing out the mode of fructification of moulds which can then be studied satisfactorily with high powers.

For the study of the morphology of Monilia in cultures, Boggs used stab cultures in 15% gelatin. The growth in the tube was hardened in 10% formalin, the glass cracked off and sections of the gelatin column cut across at any desired level. These blocks were sectioned with the freezing microtome; stained in dilute aqueous fuchsin (1 to 30) for several hours; then differentiated in saturated solution of citric acid until nearly decolorized. The sections were floated on slides, air-dried without blotting, cleared in xyol and mounted in balsam.

For staining fungi in sections of tissue Busse recommends the following method: r. Haematoxylin, 10 to 15 minutes; then wash in tap water. 2. Carbol fuchsin (1 to

20), 30 minutes or over night. Decolorize in alcohol for a few minutes, then pass through absolute alcohol and xylol to mount in balsam. The moulds are red.

The diagnosis of the mycoses is practically always a matter for the laboratory. The main dependence is upon the microscopic method backed up by culture. Nearly every one of the parasitic moulds will grow aerobically, though most are slow growers. Most of them prefer temperatures of from 22° to 30°C. though some of the more confirmed parasites grow better at 37°. It is perhaps unnecessary to state that we should, in studying any supposed mycosis, remember the ubiquitousness of saprophytic species of fungi and that these may grow in exudates due to lesions resulting from the action of bacteria or protozoa.

The attempt to diagnose a mycosis by the demonstration of antibodies is a waste of time. Some of the moulds which produce deep-seated or generalized infections may produce antibodies (agglutinins, precipitins, opsonins or mycolysins) but it is unlikely that those which grow in the upper layers of the skin (away from the superficial blood vessels) can do this. The chronicity of most of the mycoses would seem to lend support to the belief that antibodies are not produced. However, the case of microscopic diagnosis makes the demonstration of antibodies for this purpose unnecessary.

#### CHAPTER XII

## IMMUNITY AND HYPERSENSITIVENESS

## SEROLOGICAL METHODS

By immunity is meant the ability of the body to defend itself against injury by pathogenic micro-organisms or by their toxic products. Immunity is, as a rule, only relative, and its effectiveness depends upon the balance between the virulence of the invading organism and the resistance of the body.

The virulence of bacteria, in general, depends upon their invasive power and their ability to withstand and overcome the defensive forces of the animal body, and also upon the production of toxic substances. Virulence differs greatly with different species, with different strains of the same species, and even with the same strain under different conditions of growth. Thus a highly virulent strain may lose its virulence entirely if the culture becomes dissociated. Conversely, virulence may often be restored or enhanced by successive passages through animals. Bacteria, like animals. therefore, have a protective mechanism which enables them to resist the defensive forces of the body. The nature of this mechanism is not clear, and it probably differs with different species. Bail hypothecated the development in the animal body by pathogenic bacteria of substances, "aggressins," which acted mainly by inhibiting phagocytosis. The aggressin-like action of the specific capsular substance of virulent types of pneumococci is discussed in the section on the pneumococcus. With the loss of virulence that accompanies dissociation there is also a loss of capsule formation, and the organisms become phagocytable.

Toxins.—In many cases virulence is associated with the production of poisonous products. These are of two types, exotoxins, or true toxins, and endotoxins. *Exotoxins* are soluble substances which are elaborated by the living organisms, and set free in the body fluids or in culture media. They are for the most part unstable substances which are destroyed by heating to 60° or 70°C., and by certain chemicals. They are highly potent poisons which act in a specific, selective manner upon special tissues. After injection into an animal a definite period must elapse before symptoms appear. The injection of the exotoxin excites the

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production of an antitoxin which completely annuls the action of the toxin. The ability to form toxins may be lost in dissociated cultures. They are formed most characteristically by the diphtheria bacillus, and by tetanus and some other anaerobic, spore-forming bacilli.

The toxic action of most pathogenic organisms is due to a substance in the bacterial cell which is not liberated until the cell disintegrates from autolysis or from the action of bacteriolytic substances in the body. These endotoxins are less specific in their action, require no incubation period following their injection, and are relatively stable, resisting heating at 100°C. On injection into animals they stimulate the production of antibacterial sera which have little or no antitoxic neutralizing action.

Some organisms, such as the Shiga dysentery bacillus, produce both an endotoxin and an exotoxin. Antisera show both antibacterial and antitoxic activity. In the case of anthrax bacilli neither exotoxins nor endotoxins have been demonstrated, and the nature of their defensive mechanism is not known. It is probably developed only when growing in the tissues.

Immunity.—Immunity may be acquired actively as the result of an infection. In some cases, such as scarlet fever, the immunity is life long, while in others it is transient, as in pneumonia. In some diseases such as syphilis and tuberculosis immunity is usually insufficient to rid the body of infection, although it suffices as a rule to prevent superinfection (the penetration into the tissues and the growth of organisms in an animal already infected with the same species). The resistance to infection so acquired may be associated with specific antitoxic or antibacterial properties (antibodies) in the serum. As the immunity often persists long after such demonstrable activities have disappeared from the serum, it is probable that it depends primarily upon alterations in the cells—possibly the acquired capacity to produce the specific antibodies quickly and in abundance when exposure to the same infection again occurs.

An active immunity may be produced artificially by the injection of sublethal doses of bacterial toxins, killed cultures, or attenuated living organisms, and even of virulent living cultures. As a rule the immunity so produced is of shorter duration than that following an attack of the disease. A high degree of immunity, however, can be secured by repeated injections, especially if living virulent cultures are used. Hyperimmunization is brought about by the repeated injection of living virulent cultures or virus suspensions into animals which have recovered from a natural attack of the disease. The hyperimmune sera so produced possess a very high antibody content and protective power. They have been used prac-

tically in the protection and immunization of animals to such diseases as hog cholera and cattle plague.

Passive immunity.—If the serum of an immunized animal, or one which has recovered from a spontaneous infection, is injected into a normal animal, the latter may be passively protected from the infection. The degree of immunity so acquired is relatively less, and lasts only as long as the foreign serum remains in the circulation—to days to 3 weeks.

The substances in these immune sera upon which this protective power depends are known as *antibodies*. Such sera in test tube experiments commonly show various activities toward the corresponding organism, and different antibodies have been postulated to explain each of these activities (antitoxins, agglutinins, precipitins, bacteriolysins, opsonins, etc.). It is probable, however, that these phenomena are different manifestations of the activity of a single antibody. A serum which possesses the power of neutralizing a poison is commonly called an antitoxic serum, whereas one which acts more directly upon the organisms is termed an antibacterial serum.

Natural or inherent immunity may depend upon species or racial differences, or upon individual peculiarities. The inherent resistance of certain species of animals to organisms which are pathogenic for other species is usually a more complete immunity than are other types, but even this is seldom absolute. Thus the fowl, which is normally immune to tetanus, can be rendered susceptible by refrigeration, or can be killed by huge doses of the toxin. Man is naturally immune to many of the infections of animals. Natural antibodies for various bacteria occur in the serum of normal animals, usually in low concentration.

Antigen is a term applied to any substance which, when injected parenterally into a suitable animal, can stimulate the production of specific antibodies. In addition to bacteria and their toxins, practically any foreign protein, whether animal or vegetable in origin, may act as an antigen. With a few exceptions proteins from the same species of animal are not antigenic. In general only proteins possess antigenic power. Some relatively simple chemical substances, however, such as certain lipoids, carbohydrates and even drugs, which are not by themselves antigenic, may, if combined with any protein, become capable of stimulating the formation of antibodies. These react specifically with the non-protein element of the complex, but not with the protein itself. Such partial antigens are termed haptenes. They determine the specificity of the antigen. The polysaccharides of the pneumococcus are typical examples.

In general, antibodies are specific for the species of organism or antigen used to stimulate their formation. Frequently, however, they will also act to some extent on some biologically related species (group reactions). Thus a typhoid antiserum will often agglutinate the paratyphoid bacilli to some extent, and an anti-sheep precipitating serum will also precipitate goat or ox serum. This action is due to the presence of common antigenic factors in the two species in addition to antigens which are distinct and specific for each.

Occasionally an antibody will act upon some entirely unrelated species, as in the agglutination of proteus X19 bacilli by the serum of patients with typhus fever. There is evidently some common antigenic factor in the Rickettsia prowazeki and in the proteus X19. Such antigens are spoken of as heterophile antigens and the corresponding antibodies as heterophile antibodies. The Forssmann antigens constitute another widely distributed group of heterophile antigens, which contain a common lipoid haptene combined with various proteins. On injection into rabbits they stimulate the production of antibodies which cause agglutination of sheep red blood cells. The antigen has been found in the organs of various animals (horse, guinea pig, mouse, pigeon) and in various bacteria (dysentery, paratyphoid etc). A related antigen is present in the (unidentified) organism causing infectious mononucleosis since sera from these cases agglutinate sheep red blood cells in high dilutions.

Serum diagnosis.—The diagnosis of an infection may be established by the demonstration in the patient's serum of one or more of these antibody activities against a known bacterium. Conversely, we may identify an unknown organism by observing its reaction with a known immune serum. Such sera may be obtained from convalescents, but more potent sera may be easily prepared by immunizing animals. Various other protein substances may be used as antigens, and the sera obtained are used to identify an unknown protein. Rabbits are used for the production of immune sera for diagnostic purposes, while for therapeutic sera larger animals are employed. A species of organism may possess several antigenic constituents, and, for the preparation of therapeutic sera particularly, the antigens must be so prepared that all are present.

Preparation of antitoxins.—These sera are used chiefly for therapy. Beginning with a sublethal dose increasing amounts of potent toxin are injected subcutaneously. In most cases it is necessary to reduce the toxicity of the preparation by the addition of antitoxin or by converting the toxin into toxoid by treating it with formalin.

Preparation of precipitating sera.—A precipitating serum is used in medico-legal work for the identification of blood stains. A rabbit is injected intravenously with

increasing amounts (2 to 10 cc.) of human serum at 5 day intervals. Five injections usually suffice. A week after the last injection the titer of the serum is determined and further injections are given if necessary. The serum should be strong enough to give a precipitate when 0.1 cc. is added to 1 cc. of a 1-1000 dilution of human serum. (See medico-legal test for blood.) Precipitating sera may be prepared for other foreign proteins or bacteria in the same way with clear solutions of the antigen.

Preparation of agglutinating and lytic sera.—Rabbits may be injected intraperitoneally or preferably intravenously. The organisms are grown on suitable solid media, and suspensions are made in salt solution. These are killed by heating to 60°C. or by the addition of formalin as described under vaccines. Five injections are given at 5 day intervals. The dosage to be employed varies somewhat with the toxicity of the species used. The following amounts are suitable for most bacteria, such as the typhoid-dysentery group, cholera, etc. 1st dose, 1 loopful. 2nd dose, 2 loopfuls. 3rd dose, 4 loopfuls. 4th dose, 6 loopfuls. 5th dose, 1 agar slant. This may be followed by a similar amount of live organisms. One week after the last injection the titer of the serum is determined with blood from an ear vein, and if it is sufficiently high (1-1500 or over), the animal is bled from the heart with a sterile syringe and needle or other aspirating device. Twenty-five to fifty cc. of blood may be obtained in this way. The blood is allowed to clot, and the clear serum is removed. The serum is preserved by adding 0.5% phenol or 50% glycerin. If badly contaminated, it may be passed through a Berkefeld filter. It remains active for months if kept in the ice box. Complement disappears after a few days, and for bacteriolytic experiments it must be reactivated with fresh normal serum. In order to secure sera of maximal protective power, as with the pneumococcus, it is essential to use living cultures of high virulence for the last injections.

Antibody production varies with different rabbits, and it is desirable to immunize several at one time to obtain a potent serum. Occasionally an animal may die suddenly after an injection with symptoms resembling those of anaphylactic shock when intravenous injections are given. If intraperitoneal administration is used this does not occur, but the doses given should be larger and should be followed with living organisms. The method for preparing haemolytic serum is described in detail in the section on the Wassermann reaction.

Antitoxic serum combines with and neutralizes the specific toxin molecule in vitro and in vivo, so that in appropriate quantities the mixture is non-toxic. The antitoxin is produced by the body cells in response to their injury by the toxin, and is produced in such excess that free antitoxin appears in the circulation. By combining with the toxin in the circulation it prevents the toxin from further injuring the body cells. Antitoxins are also produced in response to the injections of other soluble poisons, such as snake venom and plant poisons. In such toxin-antitoxin mixtures the toxin is not destroyed, but is rendered innocuous. For instance, if a mixture of pyocyaneus toxin and antitoxin is heated, the antitoxin is destroyed, and the mixture regains its original toxicity. (Pyocyaneus toxin differs from other toxins in withstanding a temperature of roo°C.)

The exact chemical nature of the antitoxin is not known, but like other antibodies it is associated with the globulin fraction of the serum, and may be separated from the rest of the serum by precipitation with half saturated ammonium sulphate solution.

Antibacterial sera, produced by the injection of the bacterial bodies or the products of their disintegration, may manifest the following activities: bacteriolysis (haemolysis)—lysis of the cells; agglutination—clumping of the organisms; precipitation—production of turbidity or flocculi in clear solutions; opsonic activity—alteration of the organisms so that they can be ingested by phagocytes; complement fixation; and protective power, which may depend upon these, or other properties of the serum not demonstrable at present. The extent to which these various properties may be demonstrated varies with the species of organism concerned.

Bacteriolysis can be demonstrated by injecting cholera vibrios into the peritoneal cavity of a guinea pig together with a little cholera immune serum (*Pfeiffer's phenomenon*). If at intervals a drop of exudate is aspirated with a fine capillary pipette, actual disintegration of the vibrios can be observed.

The same phenomenon can be demonstrated in vitro if the vibrios are mixed with fresh cholera immune serum, but not if the serum is old, or if it has been inactivated by heating to 56°C. If, however, a little fresh normal serum is added, (which by itself has no effect on the vibrios) the inactivated immune serum completely recovers its power of dissolving the organisms. It is said to be reactivated. Two substances are, therefore, required for the reaction. One is a thermo-stable specific antibody, present only in the immune serum. The other is thermo-labile, and not specific, since it is present in fresh normal as well as immune serum. Furthermore, if the cholera vibrios are removed from the immune serum by centrifugalization and washed free from serum with salt solution, they will be dissolved if placed in fresh normal serum. They thus absorb from the serum the specific antibody, and become sensitized to the active substance in normal serum. To the non-specific labile substance in normal serum (Bordet's alexin) Ehrlich gave the name complement, because it was required to complete the action of the specific antibody. To the latter, the term sensitizing substance was given by Bordet. Ehrlich applied the term amboceptor to it, because he believed that it served as a coupler to attach the complement to the cell. To the portion of the cell to which the amboceptor is supposed to become attached, Ehrlich gave the name receptor. While Bordet's theory corresponds more closely to modern conceptions of the nature of the process than does Ehrlich's hypothesis, the latter is useful in furnishing a simple scheme by which the details of the process can be readily visualized.

If foreign red blood cells are injected into an animal, analogous antibodies, haemolysins, are produced, and their effects are more readily studied, since the phenomenon of haemolysis is easily visible. Like the bacteriolysins, the haemolytic antibody, or amboceptor, is stable at 56°C... and may be kept for long periods. When such a serum, inactivated by heating and suitably diluted, is added to a suspension of the homologous red blood cells, no haemolysis takes place, but the amboceptor becomes bound to the cells and sensitizes them. After the cells are washed and resuspended in isotonic salt solution haemolysis will take place on the addition of complement from some normal animal. These sensitized cells are used to determine the presence or absence of free complement in a mixture as in the Wassermann reaction and other complement fixation tests. These reactions are quantitative, and the amount of the antigen, amboceptor, and complement must be accurately adjusted in the tests. Methods for performing these tests are given in detail in the section on the Wassermann reaction.

Agglutinins.—Although Gruber and Durham first noted that cultures of the colon bacillus and the cholera vibrio would become aggregated into clumps when treated with specific serum, Widal first applied this agglutination reaction to the diagnosis of typhoid fever. In the process of agglutination in vitro the bacteria lose their motility (if motile) but are not killed. They may be cultivated in broth to which immune serum is added, and in this medium they develop in clumps or chains (Pfaundler's thread reaction). Agglutinins act upon dead as well as upon living bacteria.

Agglutinins, like other antibacterial antibodies, are relatively stable on standing and are not destroyed by inactivation. Complement is not necessary for the reaction. Ehrlich assigned to the agglutinin two active groups; a haptophore group by means of which he supposed that it became attached to the bacterial cell; and an agglutinophore group which he thought brought about the actual clumping. His views are no longer accepted, and these terms are of historical interest only. Electrolytes are necessary for agglutination; therefore the serum and bacterial suspensions must be prepared in physiological salt solution. Union of the agglutinin and the bacteria occurs without the presence of salts, but the actual aggregation involves physical changes which depend upon the action of the electrolytes.

Of great importance in the study of agglutination reactions is the recent work on variation and dissociation of bacteria. Studies have shown that many bacteria contain more than one antigenic constituent

each of which may give rise to specific antibodies. In the pneumococcus, for example, there is, in addition to the protein of the bacterial body, a carbohydrate present in the capsular material, which, when combined with the bacterial protein, gives to it a type specificity. This carbohyhydrate alone is not antigenic, but it reacts like a haptene with the antibody produced by the combined antigen. Agglutination produced by such an antigen-antibody reaction occurs in large flakes. When a strain has become dissociated by prolonged cultivation or other means into the rough (R) colony form, the capsular material is lost, and the antigenic properties of the strain are thereby altered. Agglutinating sera prepared with these R strains are no longer type-specific, and when added to an emulsion of the organisms, the resulting agglutination is of the small flake or granular type. Such cultures also tend to as spontaneousl

In the case of a motile species like the typhoid bacillus, there are at least two antigenic substances, one contained in the flagella (the H or flagellar antigen), and one in the body of the bacillus (the O or somatic antigen). Formolized suspensions contain only the H antigen, while alcoholized suspensions contain only the O antigen. The H antigen is destroyed by heat (80-100°C.), whereas the O antigen is thermostabile at this temperature. Agglutinating sera can be prepared for each individually. An anti-flagellar agglutinating serum clumps the organisms in large flakes, whereas an anti-serum to the somatic antigen causes small flake or granular agglutination. The dissociation of the typhoid bacillus from S to R form results in a change in the somatic (O) antigen, which is then designated R, or  $\not D$ , with loss of agglutinability in anti- O serum. (See section on the typhoid bacillus).

Of the other members of this group of organisms, some may possess one or another of these antigens in common. Furthermore, in some species the flagellar (H) antigen may occur in 2 forms (diphasic). In one phase it is specific, whereas in the other (group) phase, it is formed also in other related species. Identification then depends upon agglutination tests with sera prepared with each of these antigenic constituents.

For clinical purposes, however, it is usually possible to differentiate an organism from closely allied species by absorption of agglutinins from the serum. For example, a typhoid immune serum will frequently agglutinate paratyphoid as well as typhoid bacilli, but usually only in a lower dilution. An immune serum absorbed with an emulsion of the homologous organism loses all its agglutinating ability for allied strains as well. If, however, it is absorbed with a heterologous strain, only the correspond-

ing group agglutinin is removed, and the agglutinating activity for the homologous organism is but little affected. If an unknown organism, therefore, removes all agglutinins from an immune serum, we know that it is identical with the type used in its production, but if it removes only group agglutinins leaving the specific agglutinins unaffected, it must be only a related strain. By absorbing the group agglutinins from an immune serum it can be made specific.

Diagnostic use of agglutination tests.—The demonstration of specific agglutinins in a patient's serum is of particular diagnostic value in cases of typhoid and paratyphoid fevers (Widal test), brucellosis (melitensis and abortus types), tularaemia, typhus fever (using Proteus X2 and X19 as the antigens), and Rocky Mountain spotted fever (Proteus X19). Agglutinins develop in other diseases such as cholera, dysentery, etc., but only after the acute phase of the disease is over, and other bacteriological methods are more useful in diagnosis. The tests are performed in the following way.

Macroscopic agglutination tests.—Strains of known agglutinability are grown on appropriate solid media, and emulsified in a few cc. of sterile salt solution. To this suspension is added o.r% of formalin. The tubes are then stoppered tightly and placed in the ice box for 3 days, after which they should be sterile. These formolized suspensions keep for months. Live suspensions may be used equally well, but are less safe to handle. In the case of the typhoid bacillus, however, the O antigen is interfered with by the formalin and it may be necessary to use in addition an alcoholized suspension which contains the O antigen, or else a live suspension which contains both. Parallel tests should also be made with the paratyphoids. When the suspensions are sterile they are diluted with salt solution to a constant density—approximately that of tube 5 of the nephelometer tubes used in preparing vaccines.

Place in a rack 10 small test tubes of about  $\frac{3}{8}$  inch diameter. Pipette 0.9 cc. of physiological salt solution into the first and 0.5 cc. into each of the other tubes. Add to the 1st tube 0.1 cc. of the serum to be tested and mix. Transfer 0.5 cc. to the second tube, and so on through the ninth tube. Discard 0.5 cc. from this tube. One-half cc. of the bacterial suspension is then added to each of the 10 tubes. The serum dilution in the first tube is now 1-20, in the second tube 1-40, and so on. The dilution in the ninth tube is 1-5120. The tenth tube is a control without serum. Shake the tubes, and place them in a water bath at  $37^{\circ}$ C. for 2 hours. If no agglutination is evident, place them in the ice box over night and read the following morning. Shake gently and note the highest dilution in which agglutination is apparent. This dilution represents the titer of the serum. In some cases, especially with the Brucella group, agglutination may be absent in the first few tubes, and appear only in the higher dilutions (proagglutinoid zones).

In order to avoid the difficulty arising from variations in the agglutinability of different strains of typhoid bacilli, and to standardize the results of agglutination tests, Dreyer prepared "standard agglutinable emulsions" suitable for making the test, by the addition of o.1% formalin to broth cultures. These emulsions are standardized

against a particular antiserum, and any increase or decrease in the agglutinability of the emulsion is expressed as an "agglutinability factor" by which a correction can be made. The end titer of a serum is converted into a "reduced-titer" by dividing the dilution by this factor. Since these suspensions were first used it has been found that the use of formalin interfered with the O antigen and that only the large flake agglutination characteristic of the H antigen occurred. If, therefore, the agglutinins in the serum of a typhoid case are mainly of the O type, as sometimes happens, this suspension may fail to detect it. For this reason an additional suspension is now made which is kept in the form of a thick emulsion in 50% alcohol, diluting it to the required density with salt solution when performing the test. In using these standard agglutinable cultures for the antigen, therefore, one uses both formolized (H) antigen and alcoholized (O) antigen.

For the interpretation of the results of agglutination tests in various diseases see the sections on Bacteriology.

Tests for the identification of unknown bacteria with immune sera are made in the same way. Since the titer of these sera is higher than that usually found in a patient's serum, the dilutions may start with 1-50 or 1-100. Type determinations of pneumococci and meningococci are described in those sections.

Microscopic agglutination tests are less commonly used now than formerly. In this method a loopful of the bacterial emulsion is mixed with a loopful of each of several serum dilutions, and hanging drop preparations are set up. Control preparations in salt solution or in 1–10 normal serum are essential. These are placed in the thermostat for 1 hour, and examined under the high power of the microscope for clumping and loss of motility. This method is applied also to the rapid identification of colonies on culture plates.

Agglutinin absorption tests.—If a sufficient quantity of bacteria is added to an agglutinating serum, the agglutinins for that particular organism are bound to the bacteria and can be removed by separating them from the serum. The exact proportion of bacteria required varies somewhat with the titer of the serum and other factors. The following method is usually adequate to effect complete absorption. Centrifuge an emulsion of living (or killed) bacteria at high speed until they are well packed. To 1 cc. of a 1—10 dilution of the serum add 0.1 cc. of the packed bacteria. Shake well and incubate in a water bath at 37°C. for 2 or 3 hours. Free the serum from bacteria by centrifugalization. (The amount of salt solution introduced with the packed bacteria is too small to alter appreciably the dilution of the serum.) In making macroscopic agglutinations with this serum a control test should be set up with the species used to absorb it to make sure that absorption is complete.

Tests for isoagglutinins and isohaemolysins in human blood are described in Chap. XIII.

Precipitins can be demonstrated in the sera of animals immunized with bacteria, bacterial filtrates, or other antigenic substances, and are closely allied to agglutinins. When such sera are mixed with a clear filtrate of the homologous antigen, visible precipitates are formed. This action is due to the coating of minute particles of the antigen with the globulin with which the antibody is associated, and subsequent aggregation and flocculation of the combination. The reaction is specific for the protein used in the immunization. Group precipitins, analogous to group agglutinins, occur for closely related species of bacteria. Normal sera seem never to contain precipitins,

Clinically the precipitin test is less often used than the agglutination test. It may be obtained in the spinal fluid in cases of meningococcus meningitis when the organisms cannot be obtained in cultures. In pneumonia due to one of the fixed types the specific soluble substance (carbohydrate) may be demonstrated in the urine, and in the sputum, by the precipitin test with the homologous immune serum. The details of these tests are described in the sections on these bacteria.

The technique of bacterial precipitin tests is similar to that of macroscopic agglutinations, but relatively low dilutions of the immune serum must be used—undiluted, 1-2, 1-4, 1-8. To 1 cc. of these dilutions an equal volume of clear bacterial filtrate is added. Control tests should be made with normal serum. The test is more sensitive if the filtrate is layered on the serum.

Medico-legal test for blood (Uhlenhuth).—The anti-human serum is titrated in the following way. In each of 4 small test tubes place 0.1 cc. of concentrated serum. Over this, layer 0.9 cc. of the serum to be tested for, in dilutions of 1-100, 1-1000, 1-10,000, and 1-20,000. If the serum is adequate in strength, a definite white ring will appear at once in tube 1, after 1 minute in tube 2, after 4 minutes in tube 3, after about 6 minutes in tube 4. The serum must give negative reactions (throughout a period of observation of 20 minutes) with salt solution, and with 1-1000 dilutions of serum from other unrelated animals.

The dried blood to be examined is scraped off, or if on a fabric the spot is cut up, and the material extracted in salt solution for several hours. A portion of the unstained fabric is treated similarly as a control. The extracts are filtered until clear, and a portion is diluted until it corresponds in protein content roughly to a 1-1000 dilution of blood (about 0.01% protein). This can be estimated by shaking the tube vigorously and noting the amount of froth. A 1-1000 dilution shows a moderate amount of froth which persists from 3 to 5 minutes. A 1-10,000 dilution does not froth. If sufficient material is available, the tests for estimating the quantity of albumin in the urine may be employed to check the dilution.

Over o.r cc. of the appropriate immune serum layer o.g cc. of the suspected extract properly diluted. If positive a ring will appear in r-2 minutes. As controls test similarly the unstained fabric extract with the same immune serum; and the extract of the unknown blood with normal rabbit serum, and preferably with 2 or 3 antisera prepared with blood of other unrelated species of animals. If these controls show no ring after 20 minutes a positive reaction is dependable. An anti-human serum will also react with the blood of most monkeys, but not of any other animals.

The test is for *protein* and not for blood as such. (See p. 698 for tests for haemoglobin and its derivatives.)

Opsonins.—Phagocytosis is one of the most important defensive mechanisms of the body against the invasion of bacteria. There are two kinds of phagocytes in the body—the microphages or polymorphonuclear leukocytes; and the macrophages which include the monocytes of the blood and the fixed tissue (reticulo-endothelial) cells. In ordinary bacterial infections the former play the predominant role. The latter are chiefly concerned in the phagocytosis of animal cells and protozoa. Although other observers had previously noted the presence of substances in immune sera which stimulated phagocytosis, Wright and Douglas in 1903 first demonstrated that these substances, which they designated opsonins, acted directly upon the homologous bacteria, rendering them susceptible to phagocytosis. These opsonins unite with the bacteria just as do other antibodies, and are not removed by washing the bacteria. The opsonins

in immune sera (bacteriotropins of Neufeld) are specific for the species used in immunization, and may be absorbed from the serum. Opsonins, active on some of the less virulent types of bacteria, occur to some extent in normal serum, but their activity is slight compared with that of the immune opsonins. They may be increased in the serum of convalescent patients. The presence of complement is apparently not essential for phagocytosis, but it accelerates the reaction greatly. Dissociated forms are more easily phagocyted than are smooth types of the same organism.

In 1902 Leishman introduced a method of determining the "phagocytic index." A mixture of leukocytes, bacteria, and patient's serum was incubated, and film preparations made. From these he counted the number of bacteria in each of a number of leukocytes and calculated the average number per leukocyte. Since, however, normal serum may contain opsonins for the organism used, Wright has modified this by obtaining in addition the phagocytic index of a normal serum as a control. By dividing the phagocytic index of the unknown serum by that of the normal serum he obtains the "opsonic index."

Wright advocated determining the opsonic index, both as a diagnostic procedure, and as an aid in guiding vaccine therapy. He regarded any notable deviation of the index from 1.0 as indicating an infection with the organism in question. A low index would indicate a decreased resistance to the organism, and a rise in the index, satisfactory improvement under treatment. There is no question as to the importance of phagocytosis as a means of defense against many types of infection, or as to the desirability of having some method of estimating its activity in a given patient. The reliability of Wright's method, however, and the validity of his conclusions have been seriously questioned. He attaches significance to changes in the index which are relatively slight. The method is open to technical errors, it is laborious and time consuming, and requires experience and skill to get consistent and dependable results. As a result it has largely fallen into disuse in this country as a clinical procedure.

Bacterial Complement Fixation.—Immune sera contain antibodies which, in the presence of the corresponding antigen, combine with complement and, in appropriate quantities, remove it from the mixture. The presence or absence of complement in the mixture is then determined by the addition of a haemolytic system (red blood cell suspension, sensitized with its specific antiserum, which has been inactivated to remove complement). Such a test is performed exactly as is the Wasserman test, with bacterial preparations as the antigen.

This test is used chiefly in the diagnosis of gonorrhea and glanders. It is of some value in tuberculosis, but is usually negative in early cases. The reaction may be obtained in a number of other infectious diseases including those due to filtrable viruses, but for practical purposes cultures or other immunity reactions are less complicated and time-consuming. It is also useful in the diagnosis of some of the animal parasitic diseases, especially *Echinococcus* infection, and infections by various trematodes and trypanosomes.

In performing the test, titrations of the antigen must be carried out as in the Wassermann reaction to determine: (1) haemolytic activity (if it is haemolytic in a dilution of more than 1-5, discard); (2) binding power, or ability to fix complement in the presence

of its anti-serum; (3) anticomplementary action, or ability to inhibit the action of complement in the absence of serum. For the actual test one should use not more than  $\frac{1}{2}$  of the maximum quantity which just fails to be anticomplementary.

Various methods for preparing the antigen have been advocated.

- 1. Emulsify the growth on agar, or special media, in salt solution, as in the preparation of vaccines. Heat the emulsion at  $60^{\circ}$ C. for an hour, and then standardize by counting or by the use of the nephelometer tubes. For the gonococcus test 4 billion per cc. makes a satisfactory antigen. This may be preserved by the addition of 0.25% trikresol or 0.5% phenol. It is important to prepare the gonococcus antigen from a mixture of a number of strains. For glanders one may use a 72 hour culture in glycerin broth, sterilized at  $60^{\circ}$ C. for 2 hours.
- 2. Besredka and Gay prepare their antigen by precipitating the saline bacterial emulsion with an equal amount of absolute alcohol. Then centrifugalize, pipette off the supernatant fluid, and dry the sediment in vacuo over sulphuric acid. The dried sediment is made into a 2% suspension with isotonic salt solution. For use this stock solution is diluted. There are also methods in which the bacterial sediment is frozen with carbon dioxide snow, and then triturated with crystals of sodium chloride so as to make an isotonic saline emulsion.
- 3. Wash off agar growth with 50% alcohol and centrifuge. Pipette off supernatant alcohol. Add 50% alcohol and let stand for 30 minutes at 37°C. and centrifuge again. Remove supernatant alcohol. Then add ether and, after shaking, let stand I hour at room temperature. If the ether is yellow, wash again with ether. Then allow to stand until sediment is perfectly dry. For use suspend one gram of the powder in 200 cc. of physiological salt solution.

Protection Tests.—The *protective power* of a serum depends upon those properties which can be demonstrated in vitro, and probably also upon other factors which cannot be determined at present. For this reason the most accurate means of ascertaining the therapeutic value of an immune serum is by testing its ability to protect animals from infection.

Protection tests for the standardization of antipneumococcus serum are usually carried out in the following way. The culture used must be of such a virulence that 0.00001 cc. of an 18 hour broth culture will kill a mouse in 48 hours. If necessary, virulence can be enhanced by passage through mice. Six dilutions of the broth culture are made in such a way that 0.5 cc. of each dilution contains varying amounts from 0.1 to 0.00001 cc. of the original culture. One-half cc. of each dilution is mixed in a syringe with 0.2 cc. of the serum, and immediately injected into the peritoneal cavity of a mouse. A control series is injected with the culture dilutions without serum. The mice are marked for identification, and the time of death of each noted. The animals which are not protected will show at autopsy enormous numbers of pneumococci in the peritoneum and in the heart's blood.

Felton has modified this procedure by using a constant dose of virulent pneumococci with varying amounts of serum, and defines as a unit that amount of serum which will protect a mouse against one million fatal doses of the culture.

### Hypersensitiveness

Hypersensitiveness is defined by Zinsser as "an increased specific reaction capacity in an individual, man or animal, to a substance which, in a normal individual of the same species, produces little or no reaction."

the various manifestations of hypersensitiveness is not thoroughly onsequence the terminology applied to the different phenome has become confused. Coca and others make a sharp distinction between, on the o hand, anaphylaxis, in which an antigen-antibody mechanism is demonstrable, and on the other hand, the group of reactions commonly termed allergies or idiosyncrasics, in which this mechanism is not so clearly apparent. In this latter group are included the manifestations of hypersensitiveness to pollens, certain drugs, bacteria, foods, etc. Wells, on the contrary, believes that sufficient relationship exists between these phenomena to permit grouping all manifestations of altered reactivity together under the term allergy. Zinsser likewise believes that the distinction between anaphylactic phenomena and allergies is artificial, and is convinced that all forms of hypersensitiveness are fundamentally identical.

For convenience in discussion, however, these manifestations may be grouped into (1) anaphylaxis in animals and in man; and (2) hypersensitiveness (allergy, atopy) to foreign sera (serum sickness), pollen and other inhaled proteins, foods, drugs, and bacteria.

Theobald Smith, studying the effects of diphtheria antitoxin in guinea pigs, and Arthus by injecting rabbits with horse serum, observed that the animals were rendered susceptible to a second injection of small amounts of the serum after an incubation period of a week or more. Various authors, notably Rosenau and Anderson, have extended these observations, and have found that the reaction could be produced by the re-injection of various proteins, and that it is specific for the particular protein used.

Anaphylactic shock.—If a guinea pig is injected parenterally with even very minute amounts of horse serum (0.0001 cc. or even less), after an interval of 10 days a condition of anaphylactic shock may be brought about by the injection of somewhat larger amounts (0.01 to 0.3 cc. intravenously or intracerebrally, or 1 to 5 cc. subcutaneously). It is assumed that the primary injection has, at the end of the requisite interval, sensitized the body cells to the particular alien protein introduced, since the administration of large amounts of serum does not affect a guinea pig on primary injection. In guinea pigs symptoms are produced within a few minutes of the second injection. Restlessness, scratching of the nose, and sometimes sneezing, appear first. Soon signs of great respiratory embarrassment occur, which are due to spasm of the bronchial musculature, and at autopsy there is a characteristic inflation of the lungs. Convulsions may occur and the animal may die within a few minutes. If the

shock is not immediately fatal, the body temperature may drop abruptly 5 or even 10°F. It has been shown that during the shock the complement in the blood is diminished, and it is believed that this plays a part in the reaction.

The phenomena differ in different species of animals, but they are always the same in any one species regardless of the antigen. In rabbits there are circulatory disturbances with a fall in blood pressure, spasm of the arterioles in the lungs, and marked distension of the right side of the heart. In the dog there is engorgement of the hepatic and splanchnic circulation with an extreme drop in blood pressure.

Desensitization.—If an animal is re-injected during the incubation period, before sensitiveness has developed, no reaction will take place, and the animal is desensitized temporarily to the subsequent injection. This condition has been termed antianaphylaxis by Besredka and Steinhardt. Similarly, if an animal recovers from an anaphylactic shock it is for a time desensitized. By repeated injection of the antigen in increasing amounts, beginning with a dose too small to induce symptoms, an animal may be desensitized also, and rendered insusceptible to amounts which would otherwise produce a fatal anaphylactic shock.

Passive sensitization.—When serum from a sensitized animal is injected into a normal animal, even in quantities as small as 0.5 cc., the latter is also rendered sensitive, but only after the lapse of a certain period of time. usually four hours. It is assumed that this period is necessary for fixation of the antibody to the cells. This passive sensitization lasts only about two weeks. The offspring of an animal sensitized before or during pregnancy is passively sensitized through the placental circulation. In this type of passively induced hypersensitiveness to foreign serum there is obviously some substance present in the serum which is capable of altering the cells of a normal animal so that they become sensitive. In other types of hypersensitiveness in human beings, such as hay fever or food allergy, it is not generally possible to demonstrate by transfer to animals a sensitizing substance in the serum. It is, however, possible to produce a passive local hypersensitiveness in other human beings by intradermal injection, and it has been suggested that the inability to sensitize animals passively with human serum is due to species differences in the tissue proteins.

The substances present in the serum of sensitized animals which bring about passive sensitization are often termed anaphylactic antibodies. The nature of these antibodies is not entirely clear. They have been regarded by some as distinct from the ordinary antibodies, but there is reason to believe that they may be identical. Precipitins can be demonstrated in the blood of animals sensitized with horse serum, and also in the

blood of human beings suffering from serum sickness after a therapeutic injection of horse serum.

Tissue Hypersensitiveness.—Because a period of incubation is required to bring about passive as well as active sensitization, and because animals remain actively sensitive after their serum has lost the power to convey sensitiveness passively to other animals, it is generally accepted that the phenomena causing the anaphylactic reaction occur within or upon the tissue cells, and not primarily in the blood stream. The symptoms of shock are believed to result from the interaction within the cells of the foreign protein and the specific antibodies which are, in part at least, precipitins. The phenomena of hypersensitiveness and anaphylactic shock are, therefore, primarily cellular rather than humoral.

Relation of Hypersensitiveness to Immunity.—It is customary to include under "immunity" those forms of altered reactivity of the tissues which are obviously beneficial to the animal, and to class under "hypersensitiveness" or "anaphylaxis" those which are immediately harmful. The fundamental processes which give rise to these reactions, however, are essentially identical. Both types depend upon the increased capacity of the body cells to fix and disintegrate foreign protein which has gained access to the tissues. Whether this is beneficial or harmful depends in large part upon the nature and quantity of the foreign protein present. If the latter be in the form of a pathogenic micro-organism-relatively minute in quantity but capable of indefinite multiplicationits immediate destruction and disintegration is life-saving, and the animal is "immune." If it be a relatively large quantity of foreign serum, e.g., which can not increase in quantity in the body, its accelerated disintegration affords no distinct advantage to the animal, but this may abruptly liberate such a large quantity of toxic disintegration products that the animal is acutely poisoned; it is "hypersensitive" or "anaphylactic." The latter occurs practically only under artificial or experimental conditions, but the penetration of micro-organisms into the tissues is a very frequent occurrence. Under natural conditions, therefore, this increased reactivity of the tissues is almost always beneficial to the animal.

Kahn's Concept of Anaphylaxis and Tissue Hyperimmunity.—Recently,\* Kahn presented experimental evidence to the effect that the concept of tissue hypersensitiveness is not tenable. According to Kahn, such phenomena as anaphylactic shock, the Arthus reaction and the allergies of man fit into a unified picture of immunity in which tissue hypersensitiveness plays no part. The basis for these phenomena will herewith be briefly summarized in accordance with Kahn's interpretation.

Basis for ana phylactic shock.—The localization of protein or bacterial antigens in the tissues with which they come into contact is an outstanding defensive mechanism in immunity. A non-immune animal lacks this localizing mechanism, and antigen injected into a tissue or micro-organisms gaining entrance into a tissue under natural conditions soon find their way into the blood stream. In an immune animal, however, an antigen injected into a tissue or micro-organisms gaining entrance into the tissue are localized in the tissue and, under favorable conditions, are destroyed locally. Should this mechanism break down in an infection and permit the micro-organisms to enter the blood stream and become widespread, the life of the animal at once becomes endangered. This mechanism, so important to the life of the animal, is antagonized suddenly when the antigen is injected directly in the blood stream, and shock is a natural sequel. The

<sup>\*</sup> Kahn, R. L.: Tissue Immunity, 1936, Charles C. Thomas, Springfield, Ill.

presence of antigen in the blood stream is apparently not in itself the basis for shock, since it is common for antigen to be present in the blood stream of immune or infected animals under natural conditions, without causing shock. Only under conditions of injection does shock occur, which must mean that the mode of injection is directly related to shock. Occasionally, shock may be produced by injecting the antigen into some tissue of an immune guinea pig instead of in the blood stream, provided a sufficient amount is injected to permit a shock dose to reach the blood stream.

Basis for Arthus reaction.—The parenteral contact of the tissue cells of a normal animal with a protein antigen leads the cells to undergo immunologic changes which enables them to detect the antigen, to differentiate it from other substances and to enter into union with it. This capability to combine with the antigen is a primary response in immunity and is possessed by all tissues (the fixed tissues, the fluids and the phagocytes) of an immune animal. If the antigen is injected into a tissue, the tissue cells combine with the antigen in the injected area and the major portion of the antigen is thereby localized. This localization of an antigen within a tissue is bound to interfere with cell metabolism and lead to local tissue injury. This injury, in turn, calls forth inflammation, and under ideal conditions, the accumulated fluids and phagocytes incident to the inflammation lead to the local destruction of the antigen. Should, however, the quantity of antigen injected in relation to the degree of immunity of the animal be such that the inflammatory response to the injection is not sufficiently marked to destroy the antigen, then free antigen will be walled in within the inflammatory area. Free antigen within an inflammatory area tends to interfere with inflammatory processes. and cause death of local tissue. The small blood vessels, instead of being filled with blood, become filled with thrombi, and with the blood supply cut off, the tissues undergo This tissue necrosis within the inflammatory area in the Arthus reaction is thus not related to tissue hypersensitiveness, but is dependent on the presence of free antigen within the inflammatory area.

The allergies of man.—According to Kahn, hay fever, food allergy, drug allergy, etc., are manifestations not of tissue hypersensitiveness but of tissue hyperimmunity. Immunity is a physiologic function and like all physiologic functions is subject to disturbances. A common functional disturbance is that of hyperactivity (hyperacidity, hyperthyroidism, etc.), and the allergies are hyperactivities of the function of immunity. The local and systemic reactions in the allergic diseases are basically of the same nature as local and systemic reactions in immunity, except that they are explosive in nature or hyperactive. Substances that are not ordinarily antigenic are antigenic to allergic persons. The incubation period, essential in immunity, may be absent in allergic persons. A dose of antigen which may be too small to exert an effect under conditions of immunity, may exert a marked effect under conditions of hyperimmunity.

This brief summary of some of Kahn's views gives a glimpse of his approach to the subject of immunity and allergy. For a more comprehensive discussion of the subject, the reader is referred to "Tissue Immunity."

Hypersensitiveness in Man.—Human beings are much less susceptible to acute anaphylactic shock than are guinea pigs and most of the lower animals, and, therefore, there is relatively little danger in the administration of a second injection of therapeutic serum even after the period of anaphylactic incubation. Serious reactions occur more frequently following

the primary injection, particularly in asthmatics and in those who are sensitive to horse emanations. In these individuals deaths have occurred within a few minutes after a single injection of serum with symptoms of respiratory embarrassment and cyanosis. Boughton has reported the death of a man who was given I minim of horse serum intravenously to desensitize him for "horse asthma." However, Park has estimated that alarming symptoms occur in only I in 20,000 individuals and fatal reactions only in one in 50,000.

Before any therapeutic serum is administered, therefore, it is important to determine whether or not an individual is sensitive to horse serum, and particularly so when there is a history of previous serum administration, or if the person has had asthma or any form of protein hypersensitiveness. The existence of hypersensitiveness may be detected by means of the intradermal or ophthalmic test.

Ophthalmic test.—A drop of horse serum diluted 1 to 10 is instilled into the conjunctival sac. If the individual is sensitive, an inflammatory reaction occurs within 15 to 30 minutes. If severe it may be controlled by the instillation of 1–1000 adrenalin solution. This test is less sensitive than the cutaneous tests, and if positive great care must be used in administering serum.

Intracutaneous test.—The serum is diluted r-ro with salt solution, and o.r cc. is injected intradermally. If sensitiveness exists an urticarial wheal will develop within ro or 15 minutes, the extent of which gives a rough indication of the degree of sensitiveness. This is a sensitive test and many individuals give a slightly positive reaction, who do not develop any systemic reaction following the serum administration. If it is negative, it is generally safe to proceed with the treatment.

Description.—Even in cases giving no reaction to the intracutaneous test it is desirable to give a preliminary subcutaneous injection of about 1 cc. one half hour before starting intravenous administration of serum. In cases giving a marked reaction to the intracutaneous test, or any reaction to the ophthalmic test, preliminary desensitization should be carried out before giving the major part of the dose. This is done by beginning with a small subcutaneous dose of o.r cc. diluted with salt solution, and doubling the amount every half hour until 1.5 cc. have been given. If no reactions have occurred it is usually safe to give the balance of the dose intramuscularly. If intravenous therapy is to be used, one may then give o.r cc. diluted with 2 cc. of salt solution slowly into a vein, repeating with doubled doses every 20 minutes until the full amount is given. The rate of increase in the doses must depend upon the individual case.

It is essential to have available a 1-1000 solution of adrenalin to control any untoward reactions such as itching, tingling, urticaria, difficulty in breathing, cyanosis, circulatory symptoms, or lumbar pain. If such occur, 1 cc. should be given immediately and repeated as often as necessary. It should be remembered that its action is transitory, and anaphylactic symptoms may recur after the immediate effects have worn off. In severe reactions it may be necessary to repeat the dose every 10 to 20 minutes for several hours. Hurst (1934) injects 3 minims at the start and 1 minim every ½ to 1 minute until the reaction is over (the "continuous method"). In one of his cases this was kept up for 1½ hours, and he believes that by this method death could be prevented in all cases of anaphylactic shock. If the serum has been injected into the tissues of the arm or leg, a tourniquet should be applied proximal to the site of injection;

and after the acute symptoms have subsided it should be loosened cautiously, for brief intervals only, until the reaction is over.

Serum sickness.—In some individuals a therapeutic injection of foreign serum is followed after from 8 to 12 days by a characteristic group of symptoms. There is usually an erythematous rash or urticaria starting first at the site of inoculation, and later becoming generalized. Fever, joint pains, and generalized glandular enlargement are common. These symptoms occur in about 10% of the cases when less than 10 cc. are given, and in about 90% when more than 50 cc. are used. They are less frequent when concentrated sera are injected. A subsequent injection of the same protein causes the same symptoms, usually within 24 hours.

Von Pirquet and Schick have ascribed these symptoms to a reaction between the horse serum which is still present in the circulation of the patient and the homologous antibodies which are being actively formed in the tissues. Precipitins for horse serum have been demonstrated in the blood of patients with serum sickness, and conditions are analogous to introducing new serum into a sensitized individual. Both antigen and antibody are present in the blood at the same time. Zinsser regards serum sickness as "the characteristic manifestation of true protein anaphylaxis in man."

Hypersensitiveness occurs to other substances such as pollen, animal emanations, and other inhaled protein material, foods, drugs, and bacteria.

Coca and others believe that this form of hypersensitiveness is inherited and exists naturally, in human beings only, and not as the result of artificial sensitization. Zinsser and others, on the other hand, believe that the hypersensitiveness results from contact with the antigen through the respiratory and alimentary tracts and perhaps through the skin. They point out that while hypersensitiveness tends to occur in certain families, it is not to the same protein as a rule, and conclude that it is the capacity for becoming sensitized that is inherited.

Passive transfer.—This type of sensitiveness cannot be regularly transferred passively to animals except in isolated instances. In 1921, however, Prausnitz and Küster found that by injecting the serum of a sensitive individual intradermally into a normal person a local hypersensitiveness could be produced. If, after from 4 to 12 hours the homologous antigen was injected into the same area, a definite local reaction developed. Injections into other areas produced no reaction—only the cells in the neighborhood of the primary injection were sensitized. This local hypersensitiveness lasts several weeks. Walzer has demonstrated that when the serum of certain food idiosyncratics was injected intradermally into normal individuals, and these subjects were fed the food to which the idiosyncratic was sensitive, reactions appeared in these injected areas. In the serum of the sensitive individuals, therefore, are

substances analogous to, if not identical with, antibodies to which Coca has given the name reagins.

Hay fever is due to an allergic sensitiveness to pollens of certain plants. The flowering period of these plants determines the seasonal prevalence of the disease. Only those plants whose pollen is air-borne are of clinical importance.

Most of the cases occurring in the late spring and early summer, both in this country and abroad, are caused by pollens from the different grasses. Plantain is also frequently concerned at this season. Cases occurring in the early spring are due to the pollens of various trees. In this country the type occurring in the late summer and early fall is due most commonly to the ragweeds. In the western states sage brush sensitiveness is not uncommon at this season. Other pollens may be responsible for individual cases, and one patient may be susceptible to several different pollens. Hypersensitiveness may be detected by means of skin tests with extracts made from different pollens, and desensitization by the injection of increasing doses of the reacting extract is very successful in relieving symptoms. Sensitiveness usually returns, however, after the treatment is stopped.

Extracts of the various pollens for testing and for treatment may be purchased, or they may be prepared by extracting the pollens in many different ways. A solution containing 2 parts of glycerin and one part of saturated salt solution may be used. Gay, in the protein clinic of the Johns Hopkins Hospital, uses Coca's solution. (NaCl 5.0 Gm., NaHCO3 2.8 Gm., phenol 4.0 Gm., water 1000 cc.) Five Gm. of the pollen are washed with ether and extracted with 100 cc. of this solution, and allowed to stand 4 days, shaking frequently. The solution is then passed through a Berkefeld filter. The extract is standardized by determining its nitrogen content. The Folin-Wu method for blood nitrogen determination may be used, taking a quantity of extract containing about 0.15 mg. of nitrogen. This solution should contain about 0.5 mg. of nitrogen per cc., or 50,000 of Noon's units. (One unit is the amount of extract derived from one millionth of a gram of pollen, and it contains in the case of timothy pollen 0.00001 mg. of nitrogen, but as their allergenic activity varies directly with their nitrogen content it is customary to standardize them on this basis.

Dilutions of the stock solution from 1-10 to 1-10,000 are used for testing and for treatment. To test, an intracutaneous injection of 0.025 cc. is given, usually of the 1-5000 dilution first, and if this is negative, with 1-1000. In some cases 1-100 may be necessary to bring out the reaction. A positive reaction is indicated by the appearance, in from 5 to 30 minutes, of an urticarial wheal surrounded by an area of erythema, and in the more marked reactions by "pseudopods." Tests may also be made by applying stronger solutions to a scratch. In a few individuals who are undoubtedly hypersensitive, negative skin reactions are obtained. In such cases the sensitiveness may be detected by the P.-K. method of passive transfer, using a normal subject who gives negative skin reactions. Even this method occasionally fails to prove the point and the clinical history will have to be the guide as to whether specific treatment should be given.

Treatment in the average case is started with 0.1 cc. of the 1-10,000 dilution (or 1-1000 dilution, if the intracutaneous test to this concentration is slight), and the dose is increased gradually to 0.9 cc., giving 6 graded doses of the 1-10,000 and 1-1000 dilu-

tions, 8 doses from the 1-100 dilution, and 9 from the 1-10 dilution. Treatment is started 8 to 10 weeks before the beginning of the season, and injections are given three times a week. If less time is available before the season starts, the doses are given more frequently. Then injections of 0.9 cc. of the 1-10 dilution are continued at 5 day intervals throughout the season. Some workers advise attaining a maximum dose much higher than this. The usual commercial treatment sets, which include only about half this number of injections, and often are not continued throughout the season, are entirely inadequate for many cases.

This scheme of dosage must be altered to suit the needs of the individual patient, reducing the dose, and subsequently increasing it more gradually, if general reactions occur. The exact dose, however, can not be gauged by the amount of local reaction to the preceding injection. Neither a local nor a constitutional reaction is necessary for successful treatment. Injections should be subcutaneous or intramuscular, and great care taken to prevent any of the extract entering a vein.

No injection for testing or for treatment should be given unless adrenal in solution is at hand, and the patient should be under observation for at least a half hour in case an alarming general reaction should occur. Especial care should be taken at the onset of the season, since general reactions are more frequent at this time, and when passing from the weaker to the stronger extracts, as from I-100 solution to I-10 solution, etc. The symptoms resemble those occurring after the administration of horse serum in sensitive individuals, and must be treated in the same way.

Usually treatment is stopped at the end of the season, but somewhat better results are obtained if the maximum dose is continued at intervals of 2 to 4 weeks throughout the year. If treatment is not begun until the season has started, benefit can often be obtained by giving daily injections of o.i cc. to o.2 cc. of i-1000 dilution, making no effort to increase the dose. Gay has found that ordinary air-conditioning methods effectively remove pollen from the air. Symptoms are usually relieved within a half hour after entering an air-conditioned room, but recur quickly after leaving it.

Asthma and other respiratory allergies may depend upon the inhalation of other protein substances. Horse dander, the hair of various animals, feathers, orris root (in cosmetics), various fungi, and a large variety of other protein materials in the air and dust in minute amounts may cause respiratory symptoms. These substances may often be identified by skin tests as described below.

It is often difficult to discover the exciting cause of these conditions since there are innumerable proteins with which an individual may come in contact; and in these cases hypersensitiveness to an extract of dust from his environment may be demonstrated. These cases may also be benefited greatly by desensitization with the specific protein if it can be identified, or with dust extracts. These may be prepared by saturating a cupful of dust with Coca's solution, and filtering after 2 days extraction. The solution is then sterilized by passing through a Berkefeld filter. A scratch test is made with this extract. If positive, one should dilute the extract and follow the procedure previously outlined for pollen extracts. If it is negative, as it usually is, an intracutaneous test with 0.025 cc. of a 1-10 dilution is given, and if this is negative with 0.025 cc. of undiluted extract. Treatment is started with 0.1 cc. of the dilution which causes a mild local

reaction, giving injections twice weekly, and gradually increasing the dose to 0.3 cc. of undiluted extract. Patients who are highly sensitive to pollens or animal dander which may be present in the dust may give violent reactions to the undiluted extract. In many cases long-standing asthma is complicated by bacterial infection, and associated with hypersensitiveness to the bacterial protein.

Food idiosyncrasies may be manifested in many different ways: urticarial eruptions or eczema (particularly in children), angioneurotic oedema, gastrointestinal disturbances which may be violent, asthma, convulsions in children, and according to some observers, attacks of migraine or epileptic seizures in predisposed individuals. The possibility of sensitization by absorption of foreign protein from the gastrointestinal tract has been demonstrated experimentally in animals.

In some cases showing these symptoms a careful history will give a hint as to the food concerned. In a certain number of the cases, it may be determined by skin tests. These may be carried out by intracutaneous injections of extracts prepared in a manner similar to that outlined for pollens and dust. More often scratch tests are employed. Material for these tests may be purchased in the form of dried powders or glycerin pastes (containing the proteins precipitated from aqueous extracts of the foods). Small superficial scratches, which just fail to draw blood, are made an inch apart on the flexor surface of the forearm, or thigh or back. A portion of the paste, or the powder moistened with a drop of N/10 NaOH, is rubbed into the scratch. A positive reaction is indicated by the appearance of a small wheal, distinctly larger than the controls, usually within 5 to 30 minutes. The advantages of this method are the simplicity of the technique, the relatively greater stability of the extracts, and the rarity of general reactions, although these may occur. It is the most practicable method for the physician doing only occasional tests. An outspoken local reaction indicates a high degree of sensitiveness, and is usually of clinical significance.

Intracutaneous tests are about 100 times as sensitive as the scratch tests, and detect slight degrees of sensitiveness which are missed by the scratch method. However, intracutaneous tests frequently give slight or moderate reactions which are of no clinical significance. Greater discrimination is needed in their interpretation, and much greater caution to avoid general reactions. The wheals are much larger than those usually obtained by the scratch method.

Among the foods to which hypersensitiveness is most frequently encountered may be mentioned cow's milk, egg, wheat, sea foods, certain fruits (tomato, orange, and strawberry), nuts, and the commoner meats. Multiple sensitization is common, and an adequate examination requires a large number of tests. The reactivity of the skin varies, and repeated tests should be tried with suspected foods if necessary.

In a considerable number of patients with food allergy, cutaneous reactions are negative. Such cases can sometimes be detected by the Prausnitz-Koch technique, and by elimination diets.

Treatment consists in avoiding the food in question if practicable. Elimination from the diet must be complete. If difficult or impracticable as in the case of wheat or milk, desensitization may often be accomplished by giving a series of injections of extracts of the food, or, more simply, by its administration by mouth. The food is completely

eliminated from the diet, and increasing, measured doses are eaten daily, starting with minute amounts which give no symptoms. It must be taken continuously or sensitiveness may recur.

In many cases of chronic urticaria, angioneurotic oedema, or eczema in adults the cause is not food allergy. Most such cases appear to be associated with nervous tension and vasomotor instability, or chronic focal infections.

Contact dermatitis is often due to local allergic reactions to substances coming into direct contact with the skin, such as fabrics, dyes, furs, cosmetics, leaves of plants, drugs, chemicals, dust, etc. In such cases the patch tests of Bloch are much more useful than ordinary cutaneous tests.

A portion of the material is placed in the center of a piece of white blotting paper 1/2-inch square, or if the substance is liquid a bit of cotton or blotting paper is moistened with it. The cotton is applied to a normal, hairless portion of the skin, covered with a piece of water-proof fabric, cellophane or waxed paper, and fastened in place with adhesive tape. The material is applied in the same concentration as that in which it comes in contact with the skin naturally. The patch is left in place for 24 hours to 4 days unless discomfort occurs. A positive reaction reproduces the lesion with the appearance of papules or vesicles, usually within 24 or 48 hours, but occasionally only after 4 to 7 days. A normal control must show no reaction. In the case of organic substances this appears to be due to a reaction to oils rather than to protein. If the offending oil cannot be avoided, purified preparations of these allergenic oils are now available for desensitization.

Drug idiosyncrasies.—Certain drugs may cause the appearance of allergic manifestations, especially after prolonged administration—quinine, salicylates, amidopyrene, morphine, luminal, phenolphthalein, arsenic preparations, etc. It is not clear how these non-protein substances can elicit such reactions.

Zinsser believes that sensitization is due to an antigen formed in the body by the union of the chemical substance with body protein. This combination of drug and protein then sensitizes like a foreign protein. The drug portion of the combination corresponds to a haptene, and allergic reactions are brought about by its administration, just as the injection of other haptenes (for instance the carbohydrate of the pneumococcus) may cause anaphylactic shock in animals sensitized with the whole antigen. Skin tests rarely detect this form of hypersensitiveness. If necessary repeated injections of very small doses of the drug may be given.

Bacterial allergy.—An allergic condition, analogous to that observed in hay fever, may arise in an individual with a chronic or repeated infections, and a skin reaction may be obtained with the homologous bacterial protein. The best known example is that of the tuberculin reaction which is discussed in the section on the tubercle bacillus.

The mechanism of this form of allergy and its relationship to immunity are still not clear. Guinea pigs may be sensitized with tuberculoprotein as with other bacterial or

foreign protein so that typical anaphylactic reactions will develop on a second injection, but these animals will not give a positive cutaneous tuberculin test. On the other hand, if they are injected with dead or living tubercle bacilli so that a tubercle develops, both a true protein anaphylaxis (with passive transfer of sensitiveness), and cutaneous sensitivity can be demonstrated. Zinsser believes that this difference in the action of the tuberculin protein is due to chemical changes which occur in the preparation of the tuberculin and which alter its antigenic nature, and not to any fundamental difference in the phenomena of allergy and anaphylaxis.

In many other infections allergic reactions to the protein of the causative organism have been demonstrated. Colon bacillus extracts frequently give a slight reaction in normal individuals.

Tests may be made by intracutaneous injections of 0.025 cc. of solutions of purified bacterial proteins, or of a vaccine diluted to contain about 100,000,000 organisms per cc. A positive reaction may be manifested by the appearance of a small urticarial wheal, or after 12 to 24 hours by an area of induration surrounded by an erythema.

The clinical conditions in which the demonstration of allergic reactions to bacteria have thus far proved to be of practical importance are chiefly chronic asthmatic bronchitis and chronic infectious arthritis. The relationship of streptococci to arthritis has been discussed in the section on the streptococcus. Skin tests may be made with various strains of haemolytic and non-haemolytic streptococci, and treatment with a vaccine prepared from a reacting strain has seemed to be of value. Bacteria isolated from the sputum of asthmatic bronchitis may be tested in the same way, and vaccine treatment instituted with the reacting species. In these cases it may be that the improvement is due to a non-specific foreign protein reaction.

Respiratory and cutaneous symptoms indistinguishable from those of allergic sensitiveness may be due simply to an unusual vaso-motor response to heat and cold.

In the dermatomycoses, chronic secondary eczematous lesions (epidermatophytids) appear which are due to an allergic reaction to the fungus protein. The hypersensitiveness can be demonstrated by intracutaneous injections or patch tests of "trichophytin," an extract prepared from the fungus (Trichophyton). Patients infected with other species belonging to this family (Gymnoascaccae) also react to trichophytin. In the case of monilia infections a specific Monilia extract (oidiomycin) must be used. Desensitizing injections are often beneficial (Sulzberger and Wise, 1932).

The presence of *Echinococcus* infection may be determined by intracutaneous injection of sterile fluid from a cyst. Positive reactions occur within a half hour. Intracutaneous tests have also been employed in the diagnosis of lymphogranuloma inguinale and chancroidal infections, and of trichinosis, filariasis and infections with other animal parasites. These are discussed in Chapters XIX and XX.

#### PREPARATION OF VACCINES

Whereas vaccines for prophylaxis are of necessity made from stock cultures, the use of autogenous vaccines, made from cultures direct from the

patient, are preferable for treatment. In this way possible antigenic differences between various strains are avoided, and the particular organism obtained is less likely to be dissociated than a strain which has been artificially cultivated for many generations. When mixed cultures are obtained, however, as in cultures from sputum, it is necessary to use judgment in the selection of the species used in preparing a vaccine. Various methods of determining the significance of an organism obtained by culture have been suggested. The agglutination of a strain by the patient's serum, or a positive skin reaction on intradermal injection of the vaccine offers presumptive evidence that the organism is concerned in the infection. Thus, if a particular streptococcus isolated from the tonsils in a case of infectious arthritis gives a definite reaction on intradermal injection, whereas other strains of streptococci do not, one may conclude that a vaccine from that strain is more likely to be beneficial than a stock vaccine. On the other hand practically every mouth harbors streptococci of different kinds which are essentially saprophytic, and a vaccine made from them might be less desirable than a stock vaccine to which hypersensitiveness could be demonstrated.

The value of vaccine therapy in various conditions is discussed elsewhere. In evaluating the results of treatment in general, however, it is probable that desensitization and a non-specific foreign protein reaction play a part as well as specific antibody formation.

Cultures for vaccines are generally made on appropriate solid media. Blood or serum agar is necessary to obtain adequate growth for streptococcus, pneumococcus, and influenza vaccines. Plain agar is preferable for the staphylococcus and the typhoidcolon group. Special media are necessary for the gonococcus, tubercle bacillus, etc. Several slants are inoculated, and incubated the minimum length of time necessary to obtain a good growth. The growth on each slant is then emulsified in 2 or 3 cc. of sterile salt solution by means of a platinum loop or small cotton swab. Each emulsion is poured into a sterile test tube. If any fragments of the medium are present they must be removed by a short centrifugalization. When blood agar is used, the emulsion is sometimes so discolored by haemoglobin that it is necessary to sediment the bacteria by thorough centrifugalization and resuspend them in fresh salt solution. Broth cultures may be similarly treated, but there is danger of losing important antigenic factors by such manipulations. If the emulsion is in clumps, the tube may be sealed and shaken vigorously for ten or fifteen minutes, preferably in a shaking machine. If necessary large clumps may be broken up with a sterile glass rod. In some cases relatively little shaking is required, whereas streptococcus vaccines may require a great deal of breaking up, and may need to be filtered through a little sterile cotton to remove the remaining aggregations.

The suspension is then immersed in a water bath at 56° to 60°C. for from ½ to r hour to kill the organisms. Cultures are than made upon suitable media from a few drops of the vaccine to ascertain whether it is sterile. They should be incubated aerobically and anaerobically for at least 2 or 3 days. All glassware and solutions must be thor-

oughly sterilized in the autoclave, and great care must be taken in the manipulations to avoid contamination by spore-bearers.

The concentrated suspension is then standardized to determine the number of bacteria present in a given volume. For great accuracy this should be done before heating, since this undoubtedly results in some autolysis, but when virulent organisms are employed it is safer to kill the vaccine first. Several methods have been devised for standardization.

Wright's method.—In a capillary pipette with a mark about ½ inch from the tip, draw up r volume of vaccine, then a small air bubble, and then blood from the finger tip to the same mark. Mix quickly, smear on a slide, and stain. Count the number of red cells and of bacteria in several areas. The number of bacteria per cc. = ratio Bacteria counted Red cells counted × 5,000,000 × 1000.

Haemacytometer method.—In this method a counting chamber is used, preferably the special Petroff bacterial counting chamber with a depth of 0.02 mm. However, an ordinary blood counting chamber may be used with a sufficiently thin cover to enable one to focus on the bacteria clearly. The vaccine is drawn up to the 0.5 mark with either the red or the white cell pipette according to the concentration of the emulsion, and then diluted with freshly filtered weak methylene blue or 0.05% dahlia in 1% formalin to the 11 (or 101) mark. Callison recommends the following diluting fluid, which promotes rapid sedimentation of the organisms. Hydrochloric acid 2 cc., bichloride of mercury (0.2%) 100 cc., and sufficient 1% aqueous solution of acid fuchsin to color a deep cherry red. This should be freshly filtered before using. The pipette is thoroughly shaken and the mixture is run into the counting chamber. The preparation must be allowed to stand at least ½ hour before counting to allow the bacteria to settle. By direct counting of the squares the number of organisms per cc. of the suspension can be calculated and dilutions made accordingly.

Nephelometric method of McFarland.—This is the simplest method, and is sufficiently accurate for most clinical purposes. If greater accuracy is necessary the results may be checked by direct counting. This method consists in comparing the opacity of the vaccine with a series of ten standard tubes containing varying amounts of barium sulphate in suspension. These tubes may be prepared as follows. To a series of 10 tubes of uniform diameter add increasing quantities of 1% C.P. barium chloride solution, starting with 0.1 cc. in the first tube, and increasing the quantity by 0.1 cc. in each succeeding tube. Then add to each tube enough 1% C.P. sulphuric acid to bring the total volume to 10 cc. When sealed these may be kept for months. Another tube of the same diameter is kept sterilized for holding the vaccine for comparison. If vaccines are made direct from broth cultures without resuspending in saline, the barium sulphate standards must be backed with a tube of sterile broth.

In such tubes the density of the different suspensions will correspond approximately, in the case of most of the ordinary bacteria, to the following number of bacteria per constant.

No.	Number of bacteria	No.	Number of bacteria
T	300,000,000	6	1,800,000,000
2	600,000,000	7	2,100,000,000
2	900,000,000	8	2,400,000,000
3	1,200,000,000	9	2,700,000,000
4 .	1,500,000,000	10	3,000,000,000
э	1 -131		

Into a sterile tube (of the same diameter as the standard tubes) put 1 cc. of the vaccine, and dilute it with a measured amount of sterile salt solution until it matches the density of one of the tubes. The tubes must be well shaken. Calculation. Number of bacteria per cc. = number corresponding to the tube matched times the dilution. For example, if 1 cc. of vaccine is diluted to 5 cc. to match tube No. 3 it contains  $900,000,000 \times 5$ , or 4,500,000,000 organisms per cc. To make from this suspension 30 cc. of vaccine containing 500,000,000 per cc., the number of cc. of vaccine to be diluted to 30 cc. will be

$$\frac{30 \times 500,000,000}{4,500,000,000} = 3.33 \text{ cc.}$$

For greater accuracy several of the prepared tubes may be checked with vaccines of known concentration. The figures given above apply to organisms about the size of the staphylococcus, streptococcus, gonococcus and colon bacillus. Vaccines of the influenza bacillus contain about 3 times as many organisms as these standards would indicate.

After standardization phenol is added to a concentration of 0.5% (or trikresol 0.25%) of the total volume for preservation. Vaccines are best kept in bottles containing a few glass beads for shaking, and capped with a rubber stopper with a thin top through which a needle may be plunged to remove each dose.

For treatment staphylococcus and brucella vaccines are prepared in a concentration of 1 billion per cc. For most of the other vaccines a concentration of 100 million is preferable. Treatment is begun with  $\frac{1}{10}$  or  $\frac{1}{10}$  of this amount subcutaneously. Subsequent injections are given at 3 to 7 day intervals, gradually increasing the dose to 100 times the initial dose. In general the doses are so adjusted as to avoid any appreciable local or constitutional reaction.

When mixed vaccines are used pure cultures of each organism are obtained, and separate vaccines are prepared and standardized. They are then combined in appropriate concentrations.

Modifications to reduce toxicity.—Sensitized vaccines were introduced by Besredka. The bacteria are mixed with immune serum, and after standing for 3 hours they are centrifugalized and the supernatant serum is removed. After washing they are emulsified in salt solution, and killed by heating in the usual way. Besredka has used living sensitized bacteria in typhoid prophylaxis, but this is not recommended at present. These sensitized vaccines appear to yield less antibodies in animals than unsensitized heated vaccines.

Formolized vaccines.—The following method has been recommended by Costa and Ramon to reduce the toxicity of vaccines of the typhoid-dysentery group (particularly of Shiga bacillus vaccines, which are ordinarily very toxic) and also of the gonococcus. To a suspension of the organisms in salt solution is added 1% of 40% formalin. The tube is corked tightly, and incubated at 37°C. for 24 to 48 hours. Sediment and wash 3 times with sterile salt solution. Test for sterility and add 0.5% phenol as a preservative. This procedure kills vegetative bacteria, and converts any soluble toxin present into toxoid.

Various other chemical substances are also used in place of heat to kill the vaccines. Some prefer to kill the organisms by allowing the emulsion to stand with 0.5% phenol or 0.25% trikresol. If this is done, the culture controls must be made in a flask of broth

to dilute the antiseptic. Living vaccines, either attenuated or of fixed virulence, are used in some conditions for prophylaxis.

Vaccines have been prepared by various methods of autolysis and digestion. In general such preparations are antigenic unless the autolysis is carried too far, but it is questionable whether they have any advantage over other types. D'Herelle and others have shown that solution of the bacteria by bacterio plage produces a preparation which is highly antigenic. In the use of such a solution it is impossible to decide whether any benefit obtained is the result of the bacteriophage, or is due to antibody stimulation.

Therapeutic use of vaccines.—For the most part vaccines are not indicated in acute infections. In certain chronic or subacute infections they may be very beneficial, particularly in furunculosis, sinus infections, otitis media, bronchitis, especially when associated with asthma, pyelitis and cystitis, chronic gonorrheal infections, infectious arthritis, and other low grade, chronic infectious processes.

The prophylactic use of typhoid vaccine, tubercle bacillus preparations, and other vaccines are discussed under their respective headings.

Typhoid vaccine has been given intravenously for the production of protein shock and fever in the treatment of chronic arthritis, and also in syphilitic cases, especially paresis and Wassermann-fast cases. Doses should be small, not over 25 or 50 million for the first dose, and should not be increased beyond 300 million.

# THE KAHN REACTION IN THE DIAGNOSIS OF SYPHILIS

The phenomenon of precipitation with syphilitic sera was observed soon after that of complement fixation, and many attempts have been made to apply this phenomenon in the development of a precipitation test for syphilis. Michaelis (1907), Porges and Meyer (1908), Jacobstahl (1910), Bruck and Hidaka (1911), Hecht (1915), Meinike (1917), Sachs and Georgi (1918), Vernes (1920) and Dryer and Ward (1921), developed such tests. Although the last four gained some recognition, none received wide acceptance.

Kahn, in 1921, began to study the precipitation phenomenon with syphilitic sera and found the following governing principles to be essential for optimum results: 1. Proper concentration of the ingredients which enter into the test. 2. Colloidal instability of the antigen suspension. 3. Correct quantitative relation between serum and antigen suspension. 4. Hastening of the union between serum and antigen reacting substances by agitation. By applying these principles and paying particular attention to the standardization of the antigen and technique, Kahn has developed a test which has attained wide use throughout the world. Aside from its apparent specificity and high sensitivity, the Kahn test possesses the practical advantage of being completed within a few minutes after mixing the ingredients.

Since the development of the Kahn test, a number of other precipitation methods have been proposed by different authors. Among the best known of this group are the

Kline (1926) and Hinton tests (1927). The Kline test consists in mixing a cholesterolized antigen suspension with serum on a slide and noting the formation of a precipitate by means of the microscope. Kline has two methods: a "diagnostic" test and a more sensitive "exclusion" test. Hinton employs a glycerinated, cholesterolized, alcoholic extract as antigen, and the precipitation results are read after incubation on the basis of the degree of clumping of the precipitated particles. Additional methods that have gained some recognition are those of Rosenthal (1929), Johns (1930), Weiss (1930), and Eagle (1932).

The problem of determining which method is more dependable than others is a difficult one. The League of Nations Health Committee made the first attempt to evaluate tests for syphilis on an international scale. The plan of the Committee was to bring together the leading authors of tests for syphilis for a competitive conference, by having these authors examine specimens of "unknown" blood and spinal fluid with their respective methods. The methods that would show the highest specificity for syphilis and the highest sensitivity in detecting this disease would obviously be the most dependable tests for practical use.

The Kahn test was represented at the two League of Nations Conferences on tests for syphilis (Copenhagen in 1928, Montevideo in 1930). That this test proved the most dependable method at these two Conferences is well known. Recently an evaluation study of tests for syphilis was undertaken by the United States Public Health Service with the cooperation of the American Society of Clinical Pathologists (1935). Thirteen serologists participated. Each specimen of blood and spinal fluid to be examined was divided into thirteen portions and mailed to the respective laboratories in each of which it was to be examined by one method only. The blood was obtained from 152 normal persons and from 415 syphilitic patients, treated and untreated. Blood was also obtained from the following non-syphilitic patients: 62 cases of cancer, 51 cases of jaundice, 53 cases of tuberculosis, 46 cases of fever; also 54 blood samples from pregnant women and 50 from menstruating women. The spinal fluid was obtained from 110 neurosyphilitic patients, treated and untreated, and from 110 non-syphilitic neurologic patients. Tests were also made on blood from 36 cases of malaria and from 50 cases of leprosy.

The table on p. 265 summarizes the results shown by the different methods on all the samples examined, with the exception of those obtained from patients with malaria and leprosy. All methods gave some false positive reactions in these two groups. It is evident from this table that the standard Kahn tests stood out not only in specificity but also in sensitivity.

Apparatus.—(1) Test-tubes for performing test (with scrum and spinal fluid) are about 7.5 cm. in length and 1 cm. in diameter. (2) Vials (with straight wall and flat bottom) for preparing antigen suspension are about 5.5 cm. in length and 1.5 cm. in diameter. (3) Pipettes: 10 cc. graduated to 0.1 cc., 1 cc. graduated to 0.01 cc., 0.6 cc. (or 0.45 cc.) graduated to 0.15 cc., 0.5 cc. graduated to 0.025 cc. (antigen suspension pipette), 0.25 cc. graduated to 0.0125 cc. (antigen suspension pipette), and 0.2 cc. graduated to 0.001 cc. (4) The test-tube rack is made of sheet copper, 3 inches wide, 11½ inches long, 2¾ inches high, and consists of three shelves, the upper and middle ones containing three rows of ten holes, each approximately half an inch in diameter. The center row of holes is offset half an inch. (5) Shaking apparatus having a speed of 275–285 oscillations per minute, with a stroke of an inch and a half. (6) Water-bath (56°C.); centrifuge and centrifuge tubes are of standard type.

Spinal fluid tests

RESULTS IN EVALUATION OF SERODIAGNOSTIC TESTS FOR SYPHILIS\* Blood tests

Serologist	Percentage of false positive re- ports in presumably non-syphi- litic cases (468)	Percentage of positive reports in syphilitic cases (415)	Percentage of false positive re- ports in presumably non-syphi- litic cases (110)	Percentage of positive reports in syphilitic cases (110)
Brem‡	0.7		Ι.Ο	89.6
Eagle	I.I	84.1	0	78.0
Hinton†	I.7	86.6	†	†
Johns	2.8	69 0	8.3	45 - 5
Kahn (standard)	0.2	5	0	92.5
Kline	0.2	76 3	1.0	89.4
Kolmer‡	0.6	75 9	0	77.8
Kurtz§	3.0	86 6	0	88.0
Lufkin & Rytz	4.3	84 7	0.9	57 - 3
Rein	0.9	85.4	0	79.1
Ruediger‡	2.,2	88.2	4.8	96.8
Weiss	0.4	69.4	5 5	74 · 3
Williams (Army)‡	0.4	65.8	3.6	86.4

\* The figures in this table are taken from-Cumming, H. S., H. H. Hazen, A. H. Sanford, F. E. Senear, W. M. Simpson, and R. A. Vonderlehr: The Evaluation of Serodiagnostic Tests for Syphilis in the United States, J.A.M.A., 106: 2083-2087, (June 8) 1035.

† Did not perform spinal fluid tests.

‡ Performed modification of complement fixation tests; all other serologists performed flocculation tests.

§ Performed Kahn presumptive test.

Performed Kline exclusion test.

Reagents for Standard Kahn Reaction.—(1) Serum. The blood specimen is centrifuged to remove clot and cells. The serum must be entirely free from cells or other particles. Previous to its use in the test, the serum is heated in a water-bath at 56°C. for thirty minutes. When serum that has been heated is kept overnight in the ice box, it is reheated for ten minutes at 56°C. before using in the test. (2) Physiologic salt solution. A solution is prepared of 0.9 per cent sodium chloride (chemically pure) in distilled water. (3) Antigen.

Preparation of Antigen.—The antigen is a cholesterolized alcoholic extract of powdered beef heart which has been previously extracted with ether.

The reagents employed are: (1) Anaesthetic ether. (2) 95% alcohol. (3) Powdered beef heart. The latter is obtainable on the market (Digestive Ferments Co., Detroit). A large number of hearts enter into the preparation of each lot of this product, and a higher degree of uniformity is obtained than is possible when the powder is prepared on a smaller scale. If, however, it is desired to prepare the powdered muscle in the laboratory, it is carried out as follows: About 400 Gm. of heart muscle are cut out from at least three beef hearts and passed four times through a meat grinder. The ground material is spread into a thin layer on a porcelain platter or glass plate and dried by means of one or two revolving fans. After six or eight hours, when the exposed surface is relatively dry, the material is turned over and drying continued over night. When the layer of beef heart is in the form of a dry plate, it is broken up into small pieces and drying continued until the material is brittle. It is now ground into powder form by means of a mortar. (4) Cholesterol, C.P.

Ether extraction.—(1) 25 Gm. powder are placed in a 250 cc. Erlenmeyer flask and 100 cc. ether added. Flask is shaken intermittently for 10 minutes to keep powder in suspension. Mixture is filtered. Gentle pressure is exerted on mixture by means of spatula until practically no ether drops from funnel. (2) Moist powder is returned to flask and 75 cc. ether is added. Mixture is shaken 10 minutes and filtered as in (1). (3) Moist powder is returned to flask and 75 cc. ether 10 minutes and filtered as in (1). (4) Moist powder is returned to flask and 75 cc. ether 10 minutes and filtered as in (1). Powder dried at room temperature until free from ether odor; this should take not over thirty minutes if the material is stirred with a spatula.

Alcohol extraction.—Powder is weighed and returned to flask used above (after flask has been completely dried); 5 cc. alcohol (95 per cent) are added per gram of powder. Mixture is shaken 10 minutes, and is extracted for 3 days at room temperature (21°C.) in the dark without shaking. Mixture is shaken 5 minutes before filtration. Filtration of mixture: (1) in the same manner as ether extractions, or (2) by suction. Precaution: In order to avoid affecting the antigen by undue evaporation of alcohol, the maximum length of the filtration period for 25 grams of beef heart should be under 5 minutes; for 100 grams of beef heart, under 15 minutes. Apparatus: (a) Suction flask (size adjusted to amount of antigen being prepared). (b) Buchner funnel (size adjusted to amount of antigen being prepared). (c) Rubber stopper for flask perforated to fit stem of funnel (stopper should be covered with several layers of tin foil). (d) Pressure hose, connected to suction. (e) Filter paper cut to fit Buchner funnel. Filtered extract is kept as stock solution in dark at room temperature.

Addition of cholesterol (6 mg. of cholesterol per cubic centimeter of alcoholic extract).—Cholesterol is placed in an Erlenmeyer flask of at least twice the volume of antigen to be cholesterolized. The required amount of antigen is then added to the flask. The cholesterol is dissolved by placing flask in water bath at 56°C. or by gently rotating flask under warm tap water. The cholesterolized extract is filtered, and ready for standardization.

Fifty or 100 Gm. of powdered beef heart may be used for the preparation of antigen by employing proportionally larger flask and increased amounts of ether for the extraction. Thus, for the extraction of 100 Gm. of beef heart a 1000 cc. flask is employed and the ether amounts used in the four extractions are 400 cc., 300 cc., 300 cc. and 300 cc., respectively.

Standardization of Antigen. Purpose.—The purpose of standardizing antigen is to render it comparable in specificity and sensitiveness to standard Kahn antigen. The

standardization of antigen requires three steps: the titration of antigen, the determination of its sensitiveness, and its correction to meet standard requirements.

Procedure of antigen titration.—(1) Measure 0.8, 1.0, 1.1, 1.2, 1.3 cc. respectively of physiological salt solution into 5 standard antigen vials. (2) Measure into each of 5 similar vials 1 cc. of cholesterolized antigen. (3) Prepare 5 antigen suspensions by mixing the 1 cc. quantities of antigen with the varying amounts of salt solution, in series. Empty the salt solution into the antigen and as rapidly as possible (without waiting to drain the tube) pour the mixture back and forth 6 times. Permit the mixture to stand for 30 minutes. (4) Test for the dispersibility in salt solution of the lipid aggregates present in the antigen-salt solution suspensions after thorough mixing, as follows: a. Set up five series of 3 standard Kahn tubes to test the dispersibility of the five suspensions. b, Pipette 0.05, 0.025, and 0.0125 cc. quantities of each of the five antigen suspensions, in series, to the bottom of the tubes, using a 0.2 cc. or 0.25 cc. pipette graduated in 0.001 or 0.0125 cc. amounts. When measuring the antigen suspensions, in series, it is advisable to begin with the suspension containing the largest amount of salt solution, and end with the one containing the least amount of salt solution. will avoid carrying non-dispersible lipid aggregates from one suspension to the other. c. Add 0.15 cc. salt solution to each of the 15 tubes. d, Shake the rack of tubes for 3 minutes in a shaking apparatus at a speed of 275 to 285 oscillations per minute. If no such apparatus is available, rapid shaking by hand will approximate this speed. e, Add I cc. salt solution to the tubes containing the 0.05 cc. amounts of antigen suspension, and 0.5 cc. to the remaining tubes. Observe whether the fluids are opalescent or contain aggregates.

Interpretation of results.—When each of the 5 antigen suspensions are thus tested for the dispersibility of aggregates, it may be found that the antigen suspensions prepared by mixing antigen with the smallest amounts of salt solution contain aggregates which are not completely dispersed in additional salt solution, whereas the antigen suspensions containing the larger amounts of salt solution contain aggregates that are completely dispersed in additional salt solution. The smallest amount of salt solution which, when added to 1 cc. antigen, produces aggregates capable of complete dispersion upon the addition of further salt solution and giving an opalescent medium which is free from cholesterol crystals, is the titer of the antigen.

Determination of sensitiveness of antigen.—The titer of the antigen having been determined, the next step is to establish whether the sensitiveness of the antigen is comparable to that of standard antigen. To this end the antigen in question and standard antigen are tested simultaneously, with a number of syphilitic sera.

- 1. Preparation of syphilitic sera.—Ten sera are obtained, 8 from syphilitic patients and two from non-syphilitic individuals. Of the 8 sera, at least 6 should give weakly positive reactions, and the remaining, strongly positive reactions. If weakly reacting sera are not available, strongly reacting sera may be diluted with salt solution or with negative sera until they give weak reactions. All sera are heated for 30 minutes at 56°C. before being tested. If the sera employed have been heated for 30 minutes 24 or more hours previously, they should be re-heated for 10 minutes at the same temperature before use. Similar groups of sera are employed in all studies on antigen sensitiveness.
- 2. Testing newly prepared and standard antigen with sera.—Antigen suspensions are prepared with both antigens in accordance with their respective titers. Both suspensions are permitted to stand for 10 minutes and each is pipetted in 0.05, 0.025

and 0.0125 cc. amounts for a series of ten Kahn tests. The sera are then added in 0.15 cc. amounts. All the tests are shaken for 3 minutes at 275 oscillations per minute and, after adding the proper amounts of salt solution to each tube, the results with the two antigens are compared.

3. Interpretation of results.—If the results of the comparative tests with the two antigens are closely comparable, the new antigen probably possesses standard sensitiveness. To eliminate the possibilities of error, at least two additional series of comparative tests are carried out, and if the results are again comparable the newly prepared antigen may be considered as standard, although it is desirable to make as many comparative tests as possible with non-pooled sera before declaring an antigen standard.

Correction of antigen.—The sensitiveness of a newly prepared antigen may be greater or less than that of standard antigen. In either case it can readily be corrected to standard requirements. Two reagents are necessary for antigen correction: cholesterolized alcohol and sensitizing reagent.

- (a) Cholesterolized alcohol.—95% alcohol containing 0.6% cholesterol.
- (b) Sensitizing reagent.—I. The ether filtrate obtained in the preparation of antigen from 50 Gm. of heart muscle is refiltered to remove traces of powdered muscle, and is then evaporated with the aid of an electric fan. 2. When the volume has been reduced to about 10 cc. or less, the concentrated ether extract is transferred to a small, weighed, evaporating dish (capacity about 25 cc.), the transfer being made complete by washing out the residue into the small dish with a little ether. 3. Evaporation is continued with the aid of the fan until the ether odor is no longer detectable. 4. At this stage there may separate from the dark brown lipid mass, a few cubic centimeters of water. This water, which will be at the bottom of the evaporating dish, is removed by means of a capillary pipette. The lipid residue is brownish, semi-transparent and viscous. 5. The evaporating dish is now reweighed, and the weight of the residue determined. 6. The residue is transferred to an Erlenmeyer flask (about 100 cc. capacity). This is best accomplished with the aid of a small spatula. 7. A volume of absolute alcohol equivalent to 10 cc. per Gm. of residue is added to the flask. A small amount of this alcohol is employed for rinsing the evaporating dish. 8. Extraction is allowed to take place for 30 minutes at room temperature with frequent shaking of the flask. 0. The mixture is filtered, and the filtrate is allowed to stand at room temperature for 3 days. If a precipitate forms during this period, the solution is re-filtered. 10. The filtrate is cholesterolized with 6 mg. cholesterol per cubic centimeter, according to the usual technique. 11. The cholesterolized extract, known as "sensitizing reagent," is filtered and is then ready for use.

Correction of more sensitive antigen, by dilution with cholcsterolized alcohol.—1. Two 10-cc. amounts of the more sensitive antigen (cholesterolized) are measured into two 25-cc. Erlenmeyer flasks. 2. To one flask is added 1.0 cc. (10 per cent dilution), and to the other 2.0 cc. (20 per cent dilution) of cholesterolized alcohol. 3. The two diluted antigens are tested with 10 sera, employing standard antigen as a control. 4. If, for example, the antigen diluted 20 per cent gives the same results with these sera as standard antigen, this diluted antigen is given a check test with 10 other sera. If the check series show comparable results, then the entire amount of more sensitive antigen is accordingly corrected with 20 per cent cholesterolized alcohol.

The diluted antigen is finally declared standard after a number of additional comparative tests with standard antigen. If neither 10 nor 20 per cent alcohol dilution brings the antigen to standard sensitiveness, but approximates this sensitiveness, other per cent dilutions are tried; if the results are markedly more sensitive than standard even at the 20 per cent dilution, then the concentration method to be described is employed.

Correction of antigens more sensitive than standard, by concentration of lipids.—1. Into 2 small evaporating dishes are measured, respectively, 1 and 2 cc. amounts of the oversensitive antigen in a non-cholesterolized state. 2. By means of an electric fan, the two amounts are evaporated to dryness. This takes but a few minutes. 3. Each of the residues is dissolved in a 10-cc. amount of the oversensitive cholesterolized antigen; the modified antigens thus formed contain respectively 10 and 20 per cent more extract lipids than the original antigen. 4. The standard antigen and the two modified antigens are tested with 10 sera most of which are known to give weakly positive reactions, employing standard antigen as a control. 5. Suppose the modified antigen in which the increase in concentration of lipids is 20 per cent, gives the same results with sera as does standard antigen; the check examinations are then repeated with 10 additional sera (to eliminate the possibilities of error) and the entire amount of more sensitive antigen is accordingly concentrated to the extent of 20 per cent. The newly corrected antigen is finally declared standard after a given number of additional comparative tests with standard antigen. 6. If neither 10 nor 20 per cent increase in concentration brings the antigen to standard sensitiveness, the results obtained with these concentrations will indicate other concentrations which should be tried.

Correction of antigen less sensitive than standard.—Some antigens less sensitive than standard may be corrected by dilution with cholesterolized alcohol as described above. If this method does not yield desired results, the use of sensitizing reagent is resorted to as follows: r. Two 10-cc. amounts of the undersensitive antigen are measured into two 25-cc. Erlenmeyer flasks. 2. To one flask are added 0.05 cc. sensitizing reagent and r.o cc. cholesterolized alcohol, while to the other are added 0.1 cc. sensitizing reagent and 2.0 cc. cholesterolized alcohol. 3. The standard antigen and the two modified ones are tested with sera in the usual manner, employing standard antigen as a control. The modified antigen which gives results parallel with standard antigen is, after a sufficient number of comparative tests, declared as standard. In case no parallel results are obtained, other combinations of sensitizing reagent and cholesterolized alcohol are employed. Occasionally an antigen may be corrected by the use of sensitizing reagent without cholesterolized alcohol.

After the type and amount of correction has been experimentally determined using a small amount of antigen, the entire amount of antigen is corrected as indicated, and thoroughly tested before it is considered standard. It is believed that there should be limits to the amount of correction of antigen. No correction, whether it be in dilution with cholesterolized alcohol or in concentration of the lipids of the antigen, should exceed 25 per cent.

Standard (Diagnostic) Test with Serum.—This is a three-tube test, each tube containing a different proportion of serum and antigen suspension. In making large numbers of tests, it is well to have racks set up, tubes numbered and pipettes ready before mixing the antigen with salt solution for the test.

I. Preparation of standard antigen suspension.—This suspension is best prepared approximately five minutes before the sera are taken from the 56°C. water bath. Mix antigen with salt solution according to required

titer. Thus, if the titer is I cc. antigen plus I.I cc. physiological salt solution, mix the antigen as follows: (a) measure I.I cc. saline into a standard antigen suspension vial; (b) measure I cc. antigen into a similar vial; (c) pour the salt solution into the antigen, and as rapidly as possible (without waiting to drain the vial) pour the mixture back and forth approximately six times to insure thorough mixing; (d) allow the antigen suspension to stand for ten minutes before using. The suspension should not be used after standing thirty minutes.

One may mix more than I cc. of antigen with a proportionally larger amount of salt solution, but not much less than I cc. One cubic centimeter, when mixed with saline, will be sufficient for about fifteen tests; 2 cc. of antigen mixed with saline will be sufficient for about thirty-five tests.

- 2. Controls.—After the antigen suspension has stood ten minutes, measure 0.025 cc. into each of 3 tubes (controls) adding 0.15 cc. saline to one, 0.15 cc. negative serum to another and 0.15 cc. positive serum to the third; shake for 3 minutes, add 0.5 cc. saline to each and examine—the tubes containing positive and negative serums are controls for the sensitiveness of that particular antigen suspension. The saline control is a gauge of the opalescence of the suspension, and should contain no precipitate.
- 3. Measuring antigen suspension.—After the control tests have been completed, mix the antigen suspension well and measure 0.05, 0.025, and 0.0125 cc. amounts for each serum, delivering the suspension to the bottom of the tubes. When employing the standard rack which contains thirty tubes, measure 0.05 cc. amounts in the tubes of the first row, 0.025 cc. amounts in the tubes of the second row, and 0.0125 cc. amounts in the tubes of the third row.
- 4. Measuring serum.—The serum should be added as soon as possible after the antigen suspension has been pipetted, to avoid undue evaporation of the suspension. When examining large numbers of sera, it is well for one worker to measure the antigen suspension and for another to follow with the sera. Add 0.15 cc. serum to the 0.05, 0.025 and 0.0125 cc. amounts of antigen suspension, and shake the rack of tubes vigorously for ten seconds to insure thorough mixing of the ingredients. The rack can now be set aside until the remaining racks are ready for the regular three-minute shaking period. When examining a small number of sera, it is well to permit the serum-antigen mixtures to stand for 10 minutes at room temperature before shaking for three minutes. This step will render more uniform the examination of small and large numbers of specimens.

- 5. Shaking.—During the three-minute shaking period, it is important, not merely to agitate the rack of tubes, but to see to it that the fluid within the tubes is vigorously agitated. When shaking by hand, one may shake three one-minute periods with short rest periods. When a shaking machine is employed, its speed should be 275 oscillations per minute, with a stroke of an inch and a half. Shaking by hand should approximate this speed.
- 6. Addition of saline.—After the tests have been shaken, add I cc. saline to each tube of the first row of the rack (containing the 0.05 cc. amounts of antigen suspension) and 0.5 cc. saline to the remaining tubes. Shake tubes sufficiently to mix ingredients.
- 7. Reading of results.—Results may be read immediately after the addition of saline, but the final report should be based upon the findings after the tests have stood at room temperature 15 minutes after the addition of saline. Optimum reading conditions in each laboratory should be determined by trial. The following points will be found helpful: (a) When utilizing daylight for reading the tests, it is well to have but one source of light coming from a single window immediately in front of the reader. It will be found satisfactory to shade the upper and lower portions of the window, narrowing the source of light to a section several feet in height. Light from any other windows near the reader should be dimmed by lowering the window shades. (b) When holding the rack in front of the exposed section of the window, the definitely positive and the negative reactions are readily differentiated without lifting the tubes from the rack. (c) In case of weak reactions, examine each tube individually, lifting it several inches above the eye-level and slanting it until the fluid is spread into a thin layer. The precipitate will then become readily visible.

Those preferring magnification will find the substage mirror of the microscope helpful. Place mirror on reading table with concave surface upward. Hold the tube in slanting position two to three inches above the mirror and examine the image in the mirror. Either daylight or artificial light may be employed. One may also utilize an ordinary hand lens for reading the tests. A two- or three-fold magnification will be found satisfactory. Some workers prefer the use of a slit-light arrangement, the source of light being an electric bulb enclosed in a box which is provided with a narrow slit.

As far as possible, workers should limit themselves to one method of reading. The occasional use of magnification by readers who usually do not resort to it will be likely to affect the uniformity of their reading scale. It should be emphasized that certain highly magnifying agglutinoscopes

show particles in serum alone, and are thus unfit for use in this test. The magnification must be sufficiently low as to assure opalescent and clear-cut negative reactions, with entire freedom from visible particles.

8. Interpretation of results.—A definite precipitate suspended in a clear medium is read ++++. Proportionally weaker reactions are read +++, ++, + and  $\pm$  respectively. The final result is the average of the readings of the three tubes, as indicated in the following table.

OUTLINE OF KAHN TEST

Tube	No. 1	No. 2	No. 3	Completion of test
Serum: Antigen suspension Antigen suspension cc Serum (heated at 56°C. for thirty minutes) cc	3:1 0.05 0.15	6:1 0.025 0.15	12:1 0.0125 0.15	Tests are shaken three min- utes, I cc. salt solution is added to first tube and 0.5 cc. to other two tubes and re- sults are read. Final reading after tests have stood for Is minutes at room temperature.

Interpretation of Results

Reaction	No. 1	No. 2		Final result (average of reactions of three tubes)
	++++	++++	++++*	
	+++	++++	++++	
	- -	+++	++++	
	_ _	± -	++++	
9 10	-	± ±	++	
	_	_	+ -	

<sup>\*</sup> Weakly potent sera show most marked precipitation in the third tube because a small amount of reagin reacts best with a small amount of antigen suspension, the relatively larger amounts of suspension in the first two tubes being inhibitory to precipitation.

Strongly potent sera show ++++ precipitation in each tube; but, owing to the different amounts of antigen suspension employed, the precipitates are of unequal bulk, being greatest in the first tube and least in the last tube.

In rare instances, a reaction is obtained in which precipitation is marked in the first tube and weak or negative in the second and third tubes. In such a case it may be found that a 2:1 or 1:1 ratio of serum to antigen suspension may give strong precipitation reaction. Then the serum reaction is considered as

 Recording results.—Make a permanent record of findings in all tubes of each test at time of reading. Preferably, the tests should be read independently by two workers.

10. Procedure with less than three tubes.—If there is insufficient serum for the regular three-tube test, examine and report as follows: (a) If enough serum for two tubes, employ the lesser amounts of antigen suspension; report as a two-tube test. (b) If enough for one tube, employ the least amount of antigen suspension; report as a one-tube test. (c) If less than 0.15 cc. serum is available, a one-tube test (micro test) may be made by employing ten parts of serum to one part of antigen suspension. Thus, if 0.05 cc. of serum is available, it is employed with 0.005 cc. of antigen suspension. Report these reactions as micro tests.

Standard (Diagnostic) Test with Spinal Fluid.—In this test, the greater part of the spinal fluid globulins is precipitated by means of ammonium sulphate and redissolved in an amount of physiologic salt solution equivalent to a tenth of the original spinal fluid volume. The concentrated globulin solution thus obtained is then tested with antigen suspension.

1. Preparation of concentrated globulin solution. Reagents.—The reagents needed for the preparation of concentrated globulin solution are: (1) spinal fluid, (2) physiological salt solution, and (3) a saturated solution of ammonium sulphate. This salt must be of the highest purity (Baker's Analysed or Merck's Reagent).

Procedure.—(a) Centrifuge spinal fluid to render it free from cells and foreign particles. (b) Add 1.5 cc. of the clear fluid to a standard Kahn test-tube (7.5 by 1 cm.). (c) To the same tube add 1.5 cc. of a saturated solution of ammonium sulphate. (d) Mix fluids, covering mouth of tube with thumb (protected with rubber if desired) and shake tube back and forth vigorously. The thorough mixing of the spinal fluid and ammonium sulphate is of great importance. Place mixture in 56°C. water bath for fifteen minutes to hasten the precipitation of the globulins. (e) Centrifuge mixture at high speed for about fifteen minutes to completely throw down the precipitated globulins. (f) Remove the supernatant fluid as completely as possible. This is best accomplished with the aid of a finely drawn capillary pipette. The major amount of supernatant fluid is first withdrawn. The tube is then slanted at an angle of about 45° and the remaining fluid is withdrawn after bringing the opening of the capillary pipette to the point of contact of the globulin precipitate and inner tube wall. It will be found that the last trace of supernatant fluid can be removed by this method. (Some workers prefer to pour off the supernatant fluid and place the inverted tube in a rack having a layer of filter paper on the bottom. It will be found that after about 10 minutes standing, the fluid in the tube will be completely drained and absorbed by the filter paper.) (g) Add 0.15 cc. salt solution to the precipitate and redissolve it by gentle shaking. In adding this salt solution the point of the pipette should be lowered close to the bottom of the tube to avoid washing down the ammonium sulphate adhering to the inner wall. The globulin precipitate will dissolve readily. This globulin solution is now ready to be tested with antigen suspension.

2. Preparation of antigen suspension.—Mix salt solution with antigen in the same manner as for the test with serum, according to the antigen titer required for spinal fluid. The antigen suspension should stand ten minutes before its use in the test and should be used within thirty minutes. Control tests of the antigen suspension should be made, as outlined under "Standard Test with Serum," paragraph (2).

3. Measuring of antigen suspension.—With a 0.2 cc. pipette graduated to 0.001 cc., measure 0.01 cc. of antigen suspension to the bottom of a standard Kahn test-tube.

- 4. Measuring of concentrated globulin solution.—Measure 0.15 cc. of concentrated solution into the antigen suspension tube, using a 0.2 cc. pipette. Shake tests vigorously for 10 seconds to mix ingredients.
- 5. Controls.—Include positive and negative spinal fluid controls; also observe each concentrated globulin solution to establish that it is free from foreign particles.
- 6. Shaking.—After mixing the concentrated fluid with antigen suspension, shake test at standard speed for four minutes. This period is more desirable for spinal fluids than three minutes.
  - 7. Addition of sult solution.—Add 0.5 cc. physiologic salt solution to tube.
- 8. Reading of results.—A definite precipitate suspended in a clear medium is read ++++. Proportionally weaker precipitates are read +++, ++ and + respectively.

In practice, the spinal fluid test herewith described should be carried out in duplicate. Kahn Presumptive Test.—The presumptive test has been developed as an auxiliary method to the standard Kahn test, the main difference between the two methods being that the presumptive is more sensitive than the standard test. This increased sensitiveness is due to the fact that, in the presumptive test, "sensitized" antigen (The Kahn Test, Williams and Wilkins, Baltimore) is employed which possesses a greater degree of sensitiveness than "standard" antigen. The test was named presumptive because, in isolated instances, it may give positive reactions in cases free from syphilis.

The value of the presumptive test lies in the fact that it serves as a technical check on the standard test. The presumptive test, being more sensitive than the standard test, should be positive whenever the latter is positive and more strongly positive when the latter is weakly positive. The presumptive test is also a stronger criterion in establishing the absence of syphilis than the standard test.

Presum ptive test with scrum.—In performing this test it is essential to have available sensitized antigen. This antigen is prepared with standard antigen as a base. To standard antigen is added given amounts of sensitizing reagent and cholesterolized alcohol as described above under the standardization of standard antigen, with the exception that 1.5 to 2.5 per cent of sensitizing reagent may be required. The tests for the sensitivity of the sensitized antigen are carried out with weakly positive sera employing a known (standardized) sensitized antigen as a control.

In performing the test, the sensitized antigen is mixed with salt solution in accordance with the antigen titer. The antigen suspension is permitted to stand for 10 minutes, after which it is ready for use. The amount of suspension used in the test is 0.025 cc.; and the amount of serum is 0.15 cc. The amounts are pipetted into a standard tube, shaken for three minutes, 0.5 cc. salt solution added, and the results read. A marked precipitate is read positive and a moderate precipitate, weakly positive. A slight precipitate is considered negative.

Presumptive test with spinal fluid.—This test is carried out essentially as the standard test with spinal fluid except that sensitized antigen is employed.

Quantitative Test.—The standard test is only partially quantitative. A serum might give a ++++ reaction, or a ++++, ++, + or a doubtful  $(\pm)$  reaction. But two sera giving ++++ reactions might show marked variation in their potency. The degree of this variation may readily be determined by means of the quantitative procedure. Only

positive sera are employed. The test consists of two steps. The sera are first diluted in series with salt solution. Then, each dilution is tested with antigen suspension, and the highest dilution giving a positive precipitation reaction is the end point desired.

Dilution of positive serum with salt solution.—A series of serum dilutions with physiological salt solution is prepared so that the ratio of the volume of diluted serum to the volume of serum before dilution ranges from 5 (I part serum plus 4 parts salt solution) to 60 (I part serum plus 59 parts salt solution). The following scheme is employed:

```
Dilution Dilution
number
            ratio
  (1)
              5 = 0.2 cc. undiluted serum plus 0.8 cc. salt solution
  (2)
             10 = 0.7 cc. of (1) plus 0.7 cc. salt solution
  (3)
             20 = 0.2 cc. of (2) plus 0.2 cc. salt solution
             30 = 0.2 cc. of (2) plus 0.4 cc. salt solution
  (4)
  (5)
             40 = 0.1 cc. of (2) plus 0.3 cc. salt solution
   (6)
             50 = 0.1 cc. of (2) plus 0.4 cc. salt solution
             60 = 0.1 cc. of (2) plus 0.5 cc. salt solution
   (7)
```

Performance of test.—The serum dilutions being available, the antigen suspension is prepared in the usual manner. After standing for ten minutes the suspension is pipetted in o.or cc. amounts in each of 7 standard test tubes, depositing it at the bottom of the tubes. With an appropriate pipette, o.r5 cc. amounts of the 7 serum dilutions are added in order, beginning with the highest dilution (7), to the tubes containing antigen suspension. The racks are shaken for three minutes in the usual manner, o.5 cc. saline is added to each tube and the results are read.

If a serum gives a positive precipitation reaction with a dilution ratio of 60, still higher dilutions of serum are examined with antigen suspension until a positive reaction is no longer obtained. Higher dilutions are readily prepared by resorting to dilution (2) of which an excess is prepared.

The quantitative test with spinal fluid.—Employing spinal fluids that are known to give positive reactions, a series of dilutions are made similar to those of positive sera. The dilution range is from undiluted to 1:40. Undiluted spinal fluid is considered as equivalent to a 1:10 dilution, since the standard test is performed with a solution in which the globulin is concentrated to one-tenth the original volume. In the performance of the test, 0.01 cc. antigen suspension is mixed with 0.15 cc. of each of the spinal fluid dilutions. The mixtures are shaken for three minutes, 0.5 cc. salt solution is added to each tube and the results are read on a similar basis to the quantitative serum tests.

Many workers prefer the use of the more sensitive "sensitized" antigen rather than the standard antigen in the quantitative tests with serum and spinal fluid. Since these tests are made only in cases where syphilis is definitely established, it is believed that the use of a highly sensitive method is more desirable than the use of a conservative method.

Miscellaneous Tests. "Local" Reaction.—The Kahn test may be used in determining the serologic reactions of fluid obtained from chancres and other syphilitic lesions. After cleansing with physiologic salt solution, and under moderate pressure, fluid from chancres or other lesions is collected by means of a fine capillary pipette. This is deposited at the bottom of a small agglutination tube and centrifuged to throw down cellular matter. The clear supernatant fluid is mixed with standard antigen suspension in the proportion of 10:1. If 0.03 cc. of fluid is available, it is mixed with 0.003 cc. of antigen suspension. The tests are shaken for three minutes and 0.1 cc. salt solution added before reading the results. A definite precipitate is read positive, while freedom from a precipitate is read negative.

Aqueous humor reaction.—Fralick recently reported a series of Kahn reactions on aqueous humor. The fluid is treated similarly to spinal fluid by concentrating the globulins before performing the test.

Technical performance of the various Kahn procedures should be in the hands of workers properly trained in clinical laboratory methods.

Summary of Clinical Applications of Kahn Procedures.—The standard test with serum and spinal fluid is a conservative method in the diagnosis and treatment of syphilis and neurosyphilis. The results are reported on the basis of plus signs. The presumptive test with serum and spinal fluid is more sensitive but not so specific as the standard test. It is used as a check on the standard test. The results are reported as positive or negative. The quantitative test with serum and spinal fluid is of especial value as a serologic check on treatment. The results are reported on the basis of Kahn units.

# COMPLEMENT FIXATION IN THE DIAGNOSIS OF SYPHILIS

Wassermann, Neisser and Bruck, in 1906, were the first to utilize the phenomenon of Bordet and Gengou in the serologic diagnosis of syphilis. At first the reaction, as applied to syphilis, was thought to be a true antigen-antibody reaction. It is now well recognized, however, that the body in a syphilitic serum which reacts with the antigen is not an amboceptor, but a lipoidophilic substance which has the property of linking complement to the lipoidal antigen. The name "reagin" has been proposed for this lipoidophilic substance. A similar substance is present in the serum of patients suffering with yaws and the Wassermann reaction is just as constant in that disease as in syphilis.

# THE TECHNIC OF THE WASSERMANN TEST OF THE VETERANS' ADMINISTRATION\*

The test described herein is a modification of the original complement fixation test of Wassermann. The antigen is reinforced by 0.2 per cent cholesterin. Incubation is by means of the water bath at 37°C. It is believed that this Wassermann technic is simple and meets all of the important requirements of a serological test for syphilis. As a result of the 0.2 per cent cholesterinized reinforced antigen, the test is highly sensitive. The water bath incubation, as against the ice box incubation results in a minimum of false positives. The conflicting results due to the use of two antigens does not have to be contended with in this test. The short time consumed in its performance is an important element. The specimens of blood may be obtained in the morning, the test performed and completed, and the reports gotten out the same day.

# The Necessary Glassware and Apparatus for the Performance of the Test

Centrifuge tubes: 15 cubic centimeter ungraduated.

Centrifuge tubes: 15 cubic centimeter graduated.

Flasks, Erlenmeyer shape: 2000 cubic centimeter capacity. Flasks, Erlenmeyer shape: 500 cubic centimeter capacity.

Flasks, Erlenmeyer shape: 150 cubic centimeter capacity.

Glass cylinders: 25 cubic centimeters.

Glass cylinders: 100 cubic centimeters.

Glass cylinders: 250 cubic centimeters.

Needles, hollow, nickel-plated Lucr slips: gage 18.

Needles, hollow, nickel-plated Luer slips: gage 20.

Syringe, all-glass, Luer type: 10 cubic centimeter capacity.

Syringe, all-glass, Luer type: 20 cubic centimeter capacity.

Test-tube baskets, rectangular shape.

Pipettes: 10 cubic centimeter graduated in 1/10 cubic centimeter.

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Pipettes: 5 cubic centimeter graduated in  $1_{10}$  cubic centimeter. Pipettes: 2 cubic centimeter graduated in  $1_{10}$  cubic centimeter.

Pipettes: 1 cubic centimeter graduated in 1100 cubic centimeter.

Pipette boxes 16 inches long, 212 inches in diameter (containers for pipettes during sterilization), cylindrical in form, of copper, with tightly fitting lid.

Test tubes: 15 millimeters by 100 millimeters.

Racks, copper, for test tubes.

Glass beads.

Glass funnels.

Water bath running at 37°C. for incubation.

Inactivating water bath kept at temperature of 55°C.

Centrifuge with a speed of 1,000 revolutions per minute.

Tce box.

# Method of Cleaning Glassware

All glassware should be chemically clean and preferably sterile. To clean test tubes and flasks, empty and rinse in running tap water; wash inside and outside in soapy water; rinse several times in running tap water and invert in wire baskets. Dry in hot air oven at about 160°C.

Flasks should be plugged with cotton, and sterilized in the oven at a temperature of 160°C. for 1 hour, or until the cotton turns a light brown.

Pipettes should be placed after use in a jar or cylinder of clean water, with cotton in the bottom. To clean pipettes rinse thoroughly in running tap water, place in metal box or wire basket, and sterilize in oven.

If glassware becomes cloudy, immerse in bichromate cleaning fluid (2 parts potassium bichromate, 3 parts commercial sulphuric acid, and 25 parts water) for 24 hours. Rinse thoroughly in running tap water and proceed with the washing as before.

PREPARATION AND STANDARDIZATION OF REAGENTS OF WASSERMANN TEST

# Serum and Spinal Fluid

The blood is obtained from one of the veins at the bend of the elbow by means of a clean, dry, sterile, 18-gage needle. A rubber tourniquet is fastened around the arm above the elbow so as to distend the veins distally. The skin is sterilized by means of tincture of iodine, followed by alcohol. The sterile needle is then inserted into one of the prominent veins and about 5 cubic centimeters of blood is allowed to flow into a sterile test tube. The tourniquet is then loosened, the needle is withdrawn, and the puncture wound compressed with cotton previously moistened with alcohol. Tincture of iodine is then applied over the site of the puncture.

The centrifuge tube containing the blood should be immediately placed in the ice box, with the tube slanted so as to facilitate the separation of the serum after the blood coagulates. Both the specimen and the request form should be given the same number.

The specimens of blood are obtained on Mondays and Thursdays. The tests proper are done the same day, if possible. If not, they are to be kept in the ice box until the following morning. The tests must be completed and reports issued on Tuesdays and Fridays.

#### Inactivation of Serum

The serum should be freed of blood cells and inactivated by heating in a water bath at 55°C. for 15 minutes. This destroys the natural complement and a minimum amount of the reagin. It is believed that a half hour's inactivation results in too great a destruction of the Wassermann reacting bodies. For the test proper the patient's serum should be diluted with 0.85 per cent salt solution in the proportion of 1 part of serum to 4 parts of salt solution.

Spinal fluid should be freed of cells or sediment, and if it is blood tinged, should be inactivated in the same manner as blood serum; otherwise, no inactivation is necessary.

The largest quantity of serum (diluted 1-5) tested is 0.2 cubic centimeter; the largest quantity of spinal fluid tested is 1.0 cubic centimeter.

#### Preparation of Antigen

The antigen is made from normal, lean, beef-heart muscle which is ground and spread on a glass plate and allowed to dry. Twenty-five grams of the dried heart muscle are placed in a bottle, covered with ether, and tightly stoppered. The ether is changed frequently until it is perfectly colorless. Extraction is made at room temperature for 2 or 3 days, shaking occasionally each day. The ether is poured off, and the ground muscle is spread on a glass plate and allowed to dry. It is then finely pulverized.

One hundred cubic centimeters of absolute ethyl alcohol is added to this pulverized heart muscle (25 grams) and extraction is continued for 10 days at room temperature, with occasional shaking. This is then tiltered. The filtrate is known as the "plain alcoholic extract." To 100 cubic centimeters of this alcoholic extract is added 0.2 gram of Merck's cholesterin, this makes a 0.2 per cent cholesterinized antigen which is used in the test. When the cholesterin is completely dissolved, the extract is titrated for antigenic, anticomplementary, and hemolytic properties. This titration should be done about once a month. The cholesterinized beef-heart antigen is to be kept in a dark place at room temperature.

#### TITRATIONS OF ANTIGEN

# Preparation of antigen dilutions:

```
o.1 cc. antigen + 9.9 cc. salt solution = 1:100.

1.0 cc. antigen (1 100) + 1.0 cc. salt solution = 1:200.

1.0 cc. antigen (1 100) + 2.0 cc. salt solution = 1:300.

1.0 cc. antigen (1 100) + 3.0 cc. salt solution = 1:400.

1.0 cc. antigen (1 100) + 4.0 cc. salt solution = 1:500.

1.0 cc. antigen (1 100) + 5.0 cc. salt solution = 1:600.

1.0 cc. antigen (1 100) + 6.0 cc. salt solution = 1:700.

1.0 cc. antigen (1 100) + 7.0 cc. salt solution = 1:800.

1.0 cc. antigen (1 100) + 8.0 cc. salt solution = 1:900.

1.0 cc. antigen (1 100) + 9.0 cc. salt solution = 1:1000.
```

In preparing the above dilutions, add the salt solution to the antigen extract, drop by drop, thoroughly shaking the emulsion after each addition of salt solution. This is an important step in the preparation of the antigen dilutions.

Pool a number of known, strongly syphilitic sera obtained from patients who have never been treated, and inactivate at 55°C. for 15 minutes. Dilute 1-5 with salt solution (1 part serum and 4 parts salt solution), and set up the following titration:

Tube	Antigen, cubic centimeter	Pooled positive serum, 1-5, cc.	Complement, 2 units in o.2 cubic centimeter, cc.		Ambo- ceptor, 2 units, cc.	Corpuscle suspen- sion, 5 per cent, cc.	
I	0.2 of 1- 100	0.2	0.2	and for	0.2	0.2	place in 3°C. for
2	0.2 of I- 200	. 2	. 2		. 2	. 2	e .∓.
3	o. 2 of 1- 300	. 2	. 2	رن <u>بنا</u>	. 2	. 2	1 plac 73°C.
4	0.2 of 1- 400	. 2	, 2	gently 37°C.	. 2	. 2	
5	0.2 of 1- 500	. 2	. 2	4	. 2	. 2	and at 7
6	0.2 of 1- 600	. 2	. 2	8 8	. 2	. 2	th
7	0.2 of 1- 700	. 2	. 2	tubes ate a nutes.	. 2	. 2	tubes r bath inutes
8	o. 2 of 1- 800	. 2	. 2	b ii	. 2	. 2	
9	0.2 of 1- 900	. 2	. 2	ake inc 30 1	. 2	. 2	ike vat
10	0.2 of 1-1000	. 2	. 2	Shake incu 30 m	. 2	. 2	Shake wate 30 m

The antigenic unit is 0.2 cubic centimeter of the highest dilution of antigen producing complete inhibition of hemolysis. In the complement fixation tests 8-10 antigenic units constitute the dose. The antigen is so diluted that 0.2 cubic centimeter contains the dose. For example:

```
Antigenic unit, 0.2 cubic centimeter of 1:800. Dose (10 units), 0.2 cubic centimeter of 1:80.
```

The dose of antigen must be at least one-fifth of the anticomplementary and hemolytic titres.\*

#### Hemolytic and Anticomplementary Titrations

For the hemolytic and anticomplementary titrations prepare the following dilutions:

```
1.0 cc. antigen + 1.0 cc. salt solution = 1:2.
1.0 cc. antigen + 3.0 cc. salt solution = 1:4.
1.0 cc. antigen + 5.0 cc. salt solution = 1:6.
1.0 cc. antigen + 7.0 cc. salt solution = 1:8.
1.0 cc. antigen + 9.0 cc. salt solution = 1:10.
1.0 cc. antigen + 11.0 cc. salt solution = 1:12.
1.0 cc. antigen + 13.0 cc. salt solution = 1:14.
1.0 cc. antigen + 15.0 cc. salt solution = 1:16.
```

<sup>\*</sup> That is, the amount of antigen used in the final tests must not exceed one fifth of the smallest amount which shows either anticomplementary or haemolytic activity in the preliminary tests. (Editor.)

1.0 cc. antigen + 17.0 cc. salt solution = 1:18.
1.0 cc. antigen + 19.0 cc. salt solution = 1:20.

#### HEMOLYTIC TITRATION

Tube	Antigen, cubic centimeter	Corpuscle suspen- sion, 5 per cent, cc.	Salt solu- tion, 0.85 per cent, cc.	
I	0.2 of 1:2	0.2	0.6	
2	0.2 of 1:4	. 2	.6	37°C.
3	0.2 of 1:6	. 2	. 6	3.
4	0.2 of 1:8	. 2	. 6	at ute
5	0.2 of 1:10	. 2	.6	ath at minutes
6	0.2 of 1:12	. 2	.6	r ba 30 n
7	0.2 of 1:14	. 2	.6	r 3
8	0.2 of 1:16	. 2	.6	Water bath for 30 minu
9	0 2 of 1:18	. 2	.6	=
10	0 2 0 1:20	. 2	.6	

The hemolytic unit is 0.2 cubic centimeter of the highest dilution of antigen just beginning to produce hemolysis. The dose of antigen used in the test proper should be at least one-fifth the hemolytic unit.

# .1 nticom plementary Titration

For the anticomplementary titration a series of 10 tubes is set up, using the same dilutions of antigen as in the hemolytic titration.

Tube	Antigen, cubic centimeter	Complement, 2 units in 0.2 cubic centimeter, cc.	Salt solu- tion o.85 per cent, cc.		Ambo- ceptor, 2 units, cc.	Corpuscle suspension, 5 per cent, cc.	
I	0.2 of 1:2	0.2	0.2	> ±	0.2	0.2	
2	0.2 of 1:4	. 2	. 2	gently at min-	. 2	. 2	37°C.
3	0.2 of 1:6	. 2	. 2	e 2	. 2	. 2	~ ° ° ° ° ° ° ° ° ° ° ° ° ° ° ° ° ° ° °
4	0.2 of 1:8	. 2	. 2	ss pate 30	. 2	. 2	at
	0.2 of 1:10	. 2	. 2	tubes gincubate for 30	. 2	. 2	i th
5 6	0.2 of 1:12	. 2	. 2		. 2	. 2	ba u o
7	0.2 of 1:14	. 2	. 2	and 7°C.	. 2	. 2	ater bath at for 30 minutes.
8	0.2 of 1:16	. 2	. 2	Shake and 37°C. utes.	. 2	. 2	Water bath for 30 min
9	0.2 of 1:18	. 2	. 2	S	. 2	. 2	>
10	0.2 of 1:20	. 2	. 2		. 2	. 2	

The anticomplementary unit is 0.2 cubic centimeter of the highest dilution of antigen producing the slighest inhibition of hemolysis. The dose of antigen used in the test proper should be at least one-fifth the anticomplementary unit.

#### Amboceptor

Injection of Rabbits with Sheep Blood Corpuscles.—Young rabbits, of white or mixed color, are used for the preparation of amboceptor. The ear of the animal is shaved, washed with water followed by alcohol, and a sterile, dry, Luer syringe with a 20-gage needle is used to inoculate the sheep blood corpuscle suspension in the marginal vein of the ear. Two or more rabbits are inoculated at one time with 10 per cent sheep blood corpuscle suspension, beginning with 1 cubic centimeter and increasing by 0.5 cubic centimeter at 5-day intervals until four injections have been given. Five to seven days after the fourth injection the rabbit is bled from the marginal vein of the ear, a few drops of blood are obtained, and trial titration done. If the amboceptor is found to be of a high titre (at least 1-2,000), the animal is bled from the heart by means of a large Luer syringe, after being anesthetized. The blood is centrifugated, the clear serum is pipetted, inactivated at 55°C. for one-half hour, and placed in sterile ampoules and kept in the ice box. The amboceptor must be titrated each time preliminary to the performance of the Wassermann test. If not found hemolytic in a dilution of 1-2,000 it should be discarded.

Titration of Amboceptor.—This consists in using 0.2 cubic centimeter of 1-10 complement, and 0.2 cubic centimeter of sheep blood corpuscle suspension, 0.4 cubic centimeter of 0.85 per cent salt solution, and 0.2 cubic centimeter of varying dilutions of amboceptor, beginning with 1-100 dilution, up to 1-8,000 dilution. The tubes are incubated for 30 minutes at 37°C. at the end of which time the tube containing the highest dilution of amboceptor which shows complete hemolysis, is said to contain one unit. In the test proper, as well as in the titration of the complement, two units is the dose of amboceptor used.

TITRATION OF AMBOCEPTOR

Tube	Amboceptor, cubic centimeter	Comple- ment I-10, cc.	Salt solution, o.85 per cent, cc.	Corpuscle suspen- sion, 5 per cent, cc.	
1	0.2 of 1-100	0.2	0.4	0.2	
2	0.2 of 1-1000	. 2	.4	. 2	ن
3	0.2 of I-I200	. 2	. 4	. 2	37°C.
4	0.2 of 1-1600	. 2	. 4	. 2	
5	0.2 of 1-2000	. 2	. 4	. 2	
6	0.2 of 1-2400	. 2	. 4	. 2	bath
7	0.2 of 1-3000	. 2	. 4	. 2	q.
8	0.2 of 1-4000	. 2	.4	. 2	r 3°
9	0.2 of 1-6000	. 2	.4	. 2	Water for 30
10	0.2 of 1-8000	. 2	.4	. 2	

Shake the contents of each tube, and incubate in the water bath at 37°C. for 30 minutes. Titration of amboceptor should be done previous to the performance of the test proper. In this titration a constant quantity of 1-10 complement is used.

# Complement

The blood for the complement is obtained from three or more full-grown, healthy, male guinea pigs. Female guinea pigs should not be used because of the possibility of pregnancy; nor should immature, weak, or obviously sick animals be used for complement. These animals should not be bled more frequently than once a week for complement. The animals are anesthetized on the day of the test, and by means of a 20-gage needle inserted into the heart, blood is aspirated with a Luer syringe. About 5 cubic centimeters of blood is withdrawn from each animal. This blood is allowed to clot, and centrifugated. The sera of these animals are pooled. A small quantity of this pooled serum is diluted 1-10 with 0.85 per cent salt solution and is used in the titration of amboceptor.

Titration of Complement.—The titration of complement must be done just before the test proper, using 0.2 cubic centimeter of amboceptor dilution which contains 2 units; 0.2 cubic centimeter of varying dilutions of fresh complement; 0.2 cubic centimeter of 5 per cent sheep corpuscle suspension; 0.4 cubic centimeter of 0.85 per cent salt solution. These reagents make a total volume of 1 cubic centimeter in each tube.

Tube	Complement o.2 cubic centimeter of varying dilutions	Ambo- ceptor dilution contain- ing 2 units, cc.	Cor- puscle suspen- sion, per cent, cc.	Salt solution, o.85 per cent, cc.	
ı	0.2 of 1-20	0.2	0.2	0.4	L 1
	0.2 of 1-18				water . for
2		. 2	. 2	- 4	₩ .
3	0.2 of 1-16	. 2	. 2	- 4	ြို့
4	0.2 of 1-14	. 2	. 2	. 4	in 37°
5	0.2 of 1-12	. 2	. 2	-4	
5 6	0.2 of 1-10	. 2	. 2	. 4	a nu
7	0.2 of 1-8	. 2	. 2	- 4	D th
8	0.2 of 1-6	. 2	. 2	.4	Incubate bath at 30 minute
9	0.2 of 1-4	. 2	. 2	- 4	In

The tubes are incubated at 37°C. for 30 minutes, after which a reading is made. The tube containing the highest dilution of complement which causes complete hemolysis of 0.2 cubic centimeter of 5 per cent sheep-cell suspension in the presence of 2 units of amboceptor is said to contain 1 unit of complement. In the antigenic and anticomplementary titrations of antigen as well as in the test proper, 2 units of complement are used, this to be contained in 0.2 cubic centimeter of the complement dilution.

# Example:

- . I unit of complement 0.2 cubic centimeter of 1-20 dilution.
  - 2 units of complement 0.2 cubic centimeter of 1-10 dilution.

#### Suspension of Sheep-blood Cells

Sheep's blood is collected directly from the jugular vein, using a sterile, dry Luer syringe, and approximately 20 cubic centimeters is aspirated and allowed to run into a sterile Erlenmeyer flask containing glass beads. The flask is shaken vigorously until the blood is defibrinated. It is then passed through sterile gauze into centrifuge tubes, and diluted with physiological salt solution, and centrifugated rapidly so as to pack the cells. The supernatant fluid is pipetted off. The sediment of cells is again mixed with 0.85 per cent salt solution and centrifugated. Altogether, the cells are mixed and washed four times. The supernatant liquid is then pipetted off and discarded. The speed and duration of centrifugating should be uniform in each laboratory as based on the particular centrifuge in use. It should be sufficient to pack the washed cells firmly and evenly. The packed cells are diluted 1-20 by volume with 0.85 per cent salt solution and this constitutes the suspension of sheep-blood cells used in the test; 0.2 cubic centimeter is the quantity used in all titrations as well as in the test proper.

# Physiological Salt Solution

Chemically pure sodium chloride should be used in preparing the salt solution for diluting the various reagents which enter into the Wassermann test. A solution consisting of 0.85 per cent sodium chloride is prepared by adding 8.5 grams of the salt to 1000 cubic centimeters of distilled water. This solution should be sterilized and kept in Erlenmeyer flasks for short periods only.

#### Technic of Reaction

The various reagents having been prepared and standardized, and the titrations of both amboceptor and complement having been completed, one may proceed with the test proper. The tubes are set up in a double row. The tubes in the front row contain antigen emulsion, and the back row of tubes, the controls, are without antigen. In the front row of tubes are placed 0.2 cubic centimeter of serum, previously diluted 1-5 with 0.85 per cent salt solution; 0.2 cubic centimeter of complement, previously titrated and properly diluted with 0.85 per cent salt solution; and 0.2 cubic centimeter of antigen emulsion. In the back row of tubes are placed 0.4 cubic centimeter of serum (anticomplementary control), and 0.2 cubic centimeter of complement. With each set-up it is important to have positive and negative controls. The patients' sera are controlled for anticomplementary substances, complement for hemolysis, and antigen for anticomplementary action, with each series. Incubate for one-half hour at 37°C. in the water bath.

Make a mixture of equal parts of the 5 per cent suspension of sheep corpuscles and amboceptor solution (2 units of the latter are contained in 0.2 cubic centimeter). This is known as the sensitized sheep-cell mixture, and is placed in the ice box until the second phase of the Wassermann test.

To all the tubes are now added 0.4 cubic centimeter of the amboceptor and sheep-cell mixture (sensitized sheep-cell mixture). The tubes are then replaced in the water bath and incubated for one-half hour, with frequent shaking, at the end of which time a reading is made. All back tubes to which no antigen was added should show complete hemolysis, unless the serum is anticomplementary. All front tubes of the negative cases, as well as the negative control, should show complete hemolysis. The tubes of the positive cases, including the positive control, should show inhibition of hemolysis.

Allowing for natural amboceptor and anticomplementary bodies which may be found in sera, the degree of inhibition will depend on the amount of Wassermann reacting bodies present in the blood.

# Diagram of Wassermann Test for Syphilis

The squares represent the arrangement of tubes as seen by one looking down on the rack. Inside the squares appear the reagents in the order in which they are introduced, together with the amounts. The preliminary phase of the incubation is for 30 minutes at 37°C. Amboceptor and blood-cell mixture is then added and the tubes returned to the water bath. Incubation is continued for 30 minutes at 37°C., after which the reading is made.

#### BACK ROW

First tube <sup>1</sup>	First tube <sup>1</sup> Second tube <sup>1</sup>		Fourth tube	Fifth tube <sup>2</sup>		
Known positive serum	Inown positive serum Known negative serum		Antigen control for anticomple- mentary action	Unknown serum to be tested		
Serum 0.4 cc	Serum 0.4 cc.	No serum	No serum	Serum 0.4 cc.		
No antigen	No antigen	Salt sol. 0.4 cc.	Antigen 0.4 cc.	No antigen.		
Complement 0.2 cc	Complement 0.2	Complement 0.4	Complement 0.2	Complement 0.2		
(Incubation)	(Incubation)	(Incubation)	(Incubation)	(Incubation)		
Amboceptor	Amboceptor	No amboceptor	Amboceptor	Amboceptor		
Sheep cell	Sheep cell	Sheep cells 0.2 cc.	Sheep cell	Sheep cell		
Mixture 0.4 cc	Mixture 0.4 cc.		Mixture 0.4 cc.	Mixture 0.4 cc.		
(Incubation):	(Incubation)	(Incubation)	(Incubation)	(Incubation)		

#### FRONT ROW

First tube	Second tube	Fifth tube
Serum 0.2 cc	Complement 0.2 cc. (Incubation) Amboceptor Sheep cell Mixture 0.4 cc.	Serum 0.2 cc. Antigen 0.2 cc. Complement 0.2 cc. (Incubation) Amboceptor Sheep cell Mixture 0.4 cc. (Incubation)

<sup>1</sup> Control tubes for anticomplementary action of serum; twice the amount of serum is used.\*

<sup>2</sup> Only I unknown serum is shown in diagram above.

<sup>\*</sup> If a serum proves anticomplementary, the test must be rejected and another specimen of blood secured. A spinal fluid which is anticomplementary, however, may be regarded as giving a definitely positive reaction provided it is fresh and not contaminated. (Editor.)

# Spinal Fluid Examination

The following technic is to be used in doing the Wassermann test on specimens of spinal fluid. Varying amounts of spinal fluid are placed in tubes, beginning with 0.1 cubic centimeter in the first tube, and 1.0 cubic centimeter in the last tube. The control tube should contain 1 cubic centimeter of spinal fluid but no antigen. The other tubes should contain 0.2 cubic centimeter of the antigen emulsion, also 0.2 cubic centimeter of complement. Incubation should be for one-half hour at 37°C., to be followed by the addition of 0.4 cubic centimeter of amboceptor-blood cell mixture, in all the tubes, including the control tube. This is followed by incubation for one-half hour, accompanied by frequent shaking of the tubes. The results are then read as in the case of blood.

WASSERMANN TEST—SPINAL TEUID									
Tube	Spinal fluid, cc.	Complement 2 units, cc.	Anti- gen, cc.	Salt solu- tion, cc.		Hemoly- tic ambo- ceptor 2 units, cc.	Sheep cell suspen- sion 5 per cent, cc.		
ī	0.1	0.2	0,2		minutes	0.2	0.2	minutes °C.	
2	.2	. 2	. 2		j.	.2	,2	in.	
3	-4	. 2	. 2			. 2	.2		
4	. 5	. 2	. 2		37.	. 2	. 2	30	
5	0.1	. 2	. 2		ate	. 2	.2	at	
Spinal fluid control-6	0.1	. 2	.0		नुव "	. 2	.2	i pa	
Salt solution control—7	.0	. 2	. 2	0.5	Incubate	. 2	. 2	Incubate	

WASSERMANN TEST-SPINAL FLUID

# Reading of Tests

Results of tests on sera or spinal fluids are read after the second incubation, and are recorded as follows:

```
100 per cent fixation—Strongly positive (4 plus).
75 per cent fixation—Positive (3 plus).
50 per cent fixation—Suggestive positive (2 plus).
25 per cent fixation—Doubtful (1 plus).
No fixation—Negative.
```

# Interpretation of Wassermann Test in Subjects Not under Treatment for Syphilis

(A) Blood.—I. A single "strongly positive" or "positive" Wassermann test is insufficient for the purpose of making a diagnosis of syphilis in the absence of clinical data suggestive of syphilis. The Wassermann test should be repeated in such cases, and if the findings are confirmed, a diagnosis of syphilis may be made, especially if a suggestive history, or if clinical evidence of syphilis is elicited.

- 2. A claimant giving two or more "strongly positive" or "positive" Wassermann tests, but without any history of syphilis or signs or symptoms of the disease, should be thoroughly investigated from a serological standpoint, at one of the laboratory centers of the Veterans' Administration. Under the above conditions, a serological report of this laboratory of "strongly positive" or "positive" is evidence of a syphilitic infection.
- 3. A claimant whose blood gives a "suggestive positive" or "doubtful" result, upon two or more occasions, which results are confirmed by similar findings at one of the Veterans' Administration laboratory centers, should be made the subject of a careful clinical investigation. If no evidence of the disease is elicited, the claimant may be said not to have syphilis.
- 4. A "negative" reaction must not be regarded as proof of the absence of syphilis, bearing in mind the fact that treatment, or the ingestion of alcohol, may temporarily convert a positive Wassermann reaction to a doubtful or negative reaction.
- 5. A negative reaction of the blood, provided the claimant has not been under treatment for syphilis or has not recently partaken of alcohol, without any clinical symptoms or signs of syphilis, means that the claimant is free from syphilis.
- 6. The Wassermann test may be negative in the blood and positive in the cerebrospinal fluid.
- 7. In primary syphilis most cases examined are positive at the end of the fourth week after the appearance of the chancre. Probably 65 to 95 per cent of all primary cases will yield a positive Wassermann reaction. Not too much reliance should be placed on a negative reaction during what may be the primary stage of a presumptive syphilis.

In secondary syphilis the Wassermann test is positive in 95 to 100 per cent of the cases. In tertiary syphilis, the Wassermann test is positive in 60 to 75 per cent of the cases. In hereditary syphilis, it is positive in 90 to 100 per cent of the cases. In cerebrospinal syphilis, about 80 per cent of the patients have a positive reaction in the blood serum. Nearly all general paretics yield a positive reaction in the blood (90–100 per cent). In tabes, 50 to 75 per cent of the cases yield a positive reaction in the blood.

- (B) Spinal Fluid.—I. A Wassermann test may be positive in the cerebrospinal fluid and negative in the blood.
- 2. In paresis the Wassermann test is positive in the spinal fluid in 100 per cent of cases.
- 3. In tabes the Wassermann test is positive in the spinal fluid in 50 to 75 per cent of cases.

4. In cerebrospinal syphilis the Wassermann test is positive in the spinal fluid in about 75 to 85 per cent of cases.

# Interpretation of Wassermann Test in Subjects under Treatment for Syphilis

- (A) Blood.—r. Treatment or the ingestion of alcohol may temporarily convert a positive Wassermann reaction to a "doubtful," or "negative" reaction. It is therefore important to bear in mind that a "negative" reaction must not be regarded as proof of the absence of syphilis.
- 2. A "negative" reaction is not necessarily an indication for the cessation of antisyphilitic treatment.
- 3. A serum that yields a negative reaction may become positive after a few doses of mercury, or after a single injection of a small amount of salvarsan (provocative stimulation).
- 4. It is a safe rule to insist upon a negative Wassermann at intervals of 3 months the first year after cessation of treatment; a negative Wassermann reaction at intervals of 6 months during the second and third years after cessation of treatment; one negative Wassermann reaction 4 years and 5 years after cessation of treatment; before the patient can be pronounced free from infection.
- (B) Spinal Fluid.—I. No case of syphilis should be dismissed until a negative spinal fluid Wassermann reaction has been obtained.

#### Ice-Box Fixation

Modifications of Technique.—A great many variations from the technique described above are in use. The only important one which needs discussion here is the procedure of *ice-box fixation*. Instead of incubating the mixtures of complement, antigen and patient's serum in the water bath for 30 minutes, they are put in the ice box for some fixed period. Usually they are left over-night; or they may be left in the ice box for 4 hours and then put in the water bath for 30 minutes, after which sensitized red cells are added and the final incubation carried out. It is necessary that the same procedure be followed in titrating the antigen, which requires substantially higher dilution than for water-bath fixation.

Ice box fixation is now used in many laboratories, including that of the Johns Hopkins Hospital. It is undoubtedly a more sensitive method than fixation in the water-bath, and it is particularly useful in following the effects of treatment. For purposes of diagnosis a reaction showing only partial fixation must be interpreted conservatively. We prefer to report such reactions as "doubtful" or "inconclusive," and discard the use of the terms "one plus," "two plus," and "three plus." With this reserva-

tion the results seem to be as specific and dependable as those obtained by water-bath fixation.

A quantitative estimation of the amount of reacting substance or "reagin" in the blood ("titred Wassermann") is very important in following the result of treatment, particularly in Wassermann-fast cases. This is readily carried out by repeating the test in positive cases with a series of progressive dilutions of serum from 1 in 5 to 1 in 640. Such titrations show that a serum which gives a "four plus" positive reaction may contain from one to a hundred times that amount of reagin needed to give a "completely positive" reaction. As a result of treatment a patient might lose about 99% of the reacting substance in the serum before any effect on the Wassermann reaction would be evident if the usual routine procedure is followed. Variations from "one plus" to "three plus" would, therefore, represent relatively meaningless fluctuations within the last few per cent of the range of the original reagin-content of the serum.

Interpretation.—Because of the unavoidable possibility of human error we emphasize the importance of checking every positive reaction with a second specimen of blood. This is particularly important in the case of doubtful reactions. Repeated examinations should be made, if possible by a different method and in a different laboratory. A negative reaction is never more than presumptive evidence against the existence of syphilis. Final interpretation of the result, whether positive or negative, always requires a consideration of the history and clinical findings in each individual case. The incidence of positive reactions in the different stages of syphilis has been given. The relative frequency of negative reactions in the early primary stage of syphilis must be kept in mind, and dark-field examinations should be utilized for diagnosis. The occurrence of positive reactions in yaws, leprosy and occasionally in malaria has been discussed in Chapter VII, p. 152. It is possible that false positive reactions may also occur in trypanosomiasis and relapsing fever. Positive reactions, usually feeble and inconclusive but sometimes strong, have been reported in rare instances in a few other conditions, chiefly acute infections with high fever. These disappear promptly as the fever subsides and should not lead to errors in interpretation. In temperate climates a positive reaction repeatedly obtained by competent workers is sufficiently conclusive proof of infection to demand thorough treatment, regardless of the lack of a history or clinical evidences of syphilis.

The significance of a reaction which remains positive in spite of intensive treatment is not yet entirely clear. In many cases this is associated

with serious lesions, particularly of the central nervous system, the aorta or the viscera, and is always an indication for an exhaustive examination. In other cases no such lesions can be found, and in them it does not necessarily convey an unfavorable prognosis or indicate the need for more than the usual full course of treatment.

The relative advantages of complement fixation reactions and flocculation tests is still a matter of controversy. In competent hands either give satisfactory results (see Table, p. 265). It is desirable to use both procedures. The flocculation tests offer real practical advantages in that fresh guinea pig serum and sheep blood are not required. They are somewhat more sensitive. They also avoid the difficulties occasionally encountered with sera which are anticomplementary or which contain unusual amounts of natural anti-sheep haemolysin. In spite of their simplicity, however, they require as much technical skill and judgment for their proper performance and correct interpretation as does the Wassermann reaction.

# PART II HAEMATOLOGY

# CHAPTER XIII

# THE TECHNIQUE OF CLINICAL BLOOD EXAMINATIONS

METHODS OF OBTAINING BLOOD

For a study of the cells, either capillary or venous blood may be used. For the preparation of films capillary blood is much preferable. For most other purposes the use of oxalated venous blood is equally satisfactory, and it offers so many practical advantages that many use it as a routine procedure in all cases in which a complete examination is to be made. Blood obtained for ordinary chemical examinations may be used, and 2 cc. suffice for all the ordinary tests.

Venous blood.—To obtain accurate results with venous blood, the following precautions must be observed. The syringe and needle must be dry, or rinsed out with sterile physiological salt solution. More than a very brief period of stasis must be avoided or the counts will be too high. If a tourniquet is used, it must be released as soon as the needle enters the vein, and one must wait until normal conditions are restored before filling the syringe. A definite amount of anticoagulant must be used. Heparin (1 mg. per 5 cc. of blood) is the best, but it is too expensive for routine use. Potassium oxalate has generally been employed. If one works quickly, 2 mg. per cc. of blood suffice, and are preferable to larger amounts, but 3 mg. per cc. are permissible, and are more certain to prevent coagulation. If chemical examinations are not to be made on the same specimen, however, it is better to substitute for potassium oxalate a mixture of 6 parts of ammonium oxalate and 4 parts of potassium oxalate, since this does not cause shrinkage of the red cells. We recommend preparing oxalate tubes as follows. To each of a series of small test tubes add with a graduated pipette 0.04 cc. of a solution containing 6% ammonium oxalate and 4% potassium oxalate. Allow the fluid to evaporate in the incubator or drying oven at low temperature, and stopper. When needed, add just 2 cc. of blood from a syringe, and mix thoroughly at once.

Except for platelet counts and films, which must be made at once, the examinations may be postponed for 3 hours (for ordinary cell counts 24 hours). More time can be taken in filling the pipettes and greater precision attained with venous blood than with capillary blood, and duplicate determinations can easily be made if necessary. Immediately before filling the pipettes the blood must be thoroughly mixed, preferably by inverting the tube repeatedly and gently, so as to avoid frothing.

Capillary blood may be obtained either from the ear or finger, or, in infants, from the great toe or heel. The finger is preferable as it is easier to secure uniform satisfactory circulatory conditions there than in the ear. If the patient objects to the use of the finger tip, the area on the dorsum of the finger between the nail and the first joint may be used. The part must be warm and hyperaemic. If cold or cyanotic, the hand should first be immersed in warm water, or the ear lobe vigorously massaged. The skin is cleaned with 70% alcohol and dried. An abrupt quick stab to a depth of about 2 mm. is made with a blood lancet or some substitute instrument with a short, sharp cutting edge. We prefer a lancet of the spring-release type with a screw by means of which the depth of the puncture can be precisely adjusted. A good substitute is a Hagedorn needle, or a steel pen with one nib broken off, and the other sharpened on a fine-grained whetstone. Wright's glass needle may be used. The lancet is sterilized by dipping in alcohol and drying, or flaming, if desired. A freely flowing drop is essential, although a little gentle pressure is occasionally necessary and permissible. Greater pressure causes stasis and concentration of the blood. Violent squeezing may dilute the blood with tissue juice. The first drop or two is wiped away, and subsequent drops used for the examinations. If properly obtained, capillary blood and oxalated venous blood yield identical results except in macrocytic anaemias, in which the red cell count is often much higher in capillary blood than in venous blood.

Routine Blood Examinations.—For the average patient it often suffices to make a haemoglobin (or red cell volume) determination, a leukocyte count, and a survey of a stained film. If any abnormality in the blood is found or suspected, the examination should include also a count of the red cells, a determination of the sedimentation rate and cell volume, and a differential count. If there is anaemia, the icterus index and reticulocyte count should be included, as well as any other special examinations which may be indicated in the particular patient being studied.

Fresh Blood Preparations.—Microscopic examination of a fresh drop of blood is useful as a preliminary procedure, and if this shows no abnormality it is often possible to dispense with further examinations. Touch a freely flowing drop of blood about 2 mm. in diameter with the middle of a clean coverslip, and mount this immediately on a clean glass slide, tilting the coverslip slightly as it is lowered to prevent formation of air bubbles. The blood should spread out under the weight of the coverslip in a uniform layer, without any pressure. Seal with vaseline. Examine under the oil lens with dim illumination, taking care not to strike the coverslip with the objective, or the cells will be crushed. In the center of the preparation the red cells should be evenly distributed in a single layer, not piled up or arranged in rouleaux, and not distorted. With some experience any abnormality in the size, shape, or depth of color of the cells can be detected at once. The characteristic changes in sickle cell anaemia can be made out only in fresh preparations. A leukocytosis or leukopenia can be recognized, as well as any notable abnormality in the differential count. Such preparations are useful in the study of malarial and other parasites.

#### HAEMOGLOBIN ESTIMATION

There is no method available for the direct estimation of haemoglobin. The most important function of Hb., and, as far as known, its most constant property, is its ability to combine with O, in the proportion of one

molecule of O to each atom of iron that it contains. Since the weight of Hb. can not be determined directly, the most logical and actually the most precise method of estimating the Hb. content of the blood is to determine its *O-combining capacity*. This determination offers no great difficulty to the trained laboratory worker, but it is entirely impracticable as a routine clinical procedure. It is of fundamental importance, however, in checking the accuracy of other methods, and in the standardization of haemoglobinometers. (For technique see p. 695.)

All practicable methods of Hb. estimation are colorimetric. To eliminate the confusion due to the many different standards of normal which have been proposed by various observers, recent workers (Haden, Osgood, Wintrobe, etc.) have urged that the custom of expressing the amount of Hb. as a percentage of normal be abandoned, and that instead instruments be calibrated in Gm. of Hb. per 100 cc. of blood. Most modern instruments are so calibrated. One determines first the reading on the instrument, and also the O-combining capacity of the same specimen of blood. By dividing the latter figure (in volumes per cent) by 1.34 one obtains the number of Gm. of Hb. per 100 cc. of the blood in question. The scale of the instrument is adjusted accordingly.

Although it has been generally accepted that I Gm. of Hb. has an O-combining capacity of 1.34 cc., attempts to determine this factor accurately meet with serious technical difficulties, and the figure 1.34 is probably only roughly approximate at best. Despite this uncertainty, the present custom represents a great advance over the previous confusion, although it would doubtless be more logical, as Van Slyke points out, to calibrate instruments in terms of O-combining capacity, which can be determined precisely. Those who prefer to think of Hb. in percentages of normal can obtain the latter figure by dividing the reading in Gm. by the number of Gm. of Hb. in their accepted normal standard.

Sahli's Haemometer.—We regard this instrument or one of the recent modifications as the most practicable for routine clinical use. The original type of instrument utilizes as a standard a sealed tube containing a suspension of acid haematin corresponding to a 1% dilution of normal blood. A glass bead is included to facilitate mixing. The blood is diluted in an open tube of the same diameter so calibrated (in per cent, or in the newer instruments in Gm. per 100 cc.) that when filled to the 100% mark it holds just 2 cc.

Procedure.—(1) Invert the standard tube several times to mix. (2) To the open tube add N/10 HCl to the 10 mark (or higher). (3) From a freely flowing drop of blood, or well mixed oxalated blood fill the special capillary pipette provided just to the mark (20 cmm.) by gentle suction. (4) Thoroughly wipe off all the blood adhering to the outside of the pipette. (5) If the blood has been drawn slightly beyond the mark, bring back to the mark by stroking the tip of the pipette with the finger. (6) Expel the blood into the acid solution in the tube, rinse the pipette with the solution, and mix thoroughly. (7) Let stand (not in a bright light) for 10 minutes or longer. (8) Dilute the blood mixture with N/10 HCl (or distilled water) until the color exactly matches

that of the standard tube. Add a drop at a time as the end point is approached, mixing well with a fine glass rod after each addition. If the tube is closed with the finger tip and inverted, some of the fluid will be lost. (9) Read the level of the fluid in the diluting tube, and multiply by the correction factor of the instrument. (10) At once rinse and dry the pipette and tube, and put the instrument away out of the light.

The development of the brown color is a gradual process, proceeding rapidly at first, then more slowly. After 10 minutes about 95% of the maximum color has developed, and after an hour, practically all; but it is not absolutely complete until after about 24 hours at room temperature. At 55°C. the maximum color is obtained in 7 minutes, but heating causes some clouding of the suspension. The method chosen should be adhered to consistently and should be used in the standardization of the instrument. If the instrument is standardized for a ten minute interval and the dilution is materially delayed, subtract 2% from the corrected reading.

The advantage in the use of this instrument is that, as the solutions being compared are chemically identical, accurate readings can be obtained with any reasonably bright light. The great disadvantage is that the standard tube fades, rapidly if the instrument is carelessly exposed to sun light. Frequent restandardization is therefore imperative, at least once in 3 months, if gross errors are to be avoided.

To obviate this difficulty, the newer instruments are provided with tinted glass rods which do not fade, in place of the standard tube of acid haematin suspension. The Sahli-Hellige haemometer is one of the most satisfactory of these modifications. The color standard consists of a pair of four-sided glass prisms, between which is mounted the dilution chamber which is made with four plane surfaces. On one side this is graduated in Gm. per 100 cc., and on the opposite side in per cent, 14.5 Gm. being adopted as 100%. The procedure is exactly the same as with the Sahli instrument, except, as with all instruments using colored glass standards, a uniform source of light must be used, preferably a colorimeter lamp provided with a day-light glass window. The reading of the same blood by day light may be 10% lower than by an ordinary Mazda light. The initial standardization should not be omitted, but once obtained the correction factor does not change.

Excellent instruments, depending upon the same principle, have been devised by Haden, and by Wintrobe.

Numerous attempts have been made to find a colored fluid which would be a satisfactory substitute for the acid haematin standard solution in the original Sahli instrument, but all have either faded or deepened in color with use. Recently Osgood and Haskins have devised a permanent artificial standard by dissolving 32 Gm. of ferric sulphate and 80 mg. of chromic sulphate in water to make 100 cc. Unfortunately no two preparations have exactly the same color and each must be standardized and adjusted. Blood which is diluted 1 to 100 with N/10 HCl is compared with this solution in a Duboscq colorimeter or in comparator tubes. Correction must be made for temperature. The standard solution, with the tables necessary for conversion of the readings, may be purchased.

Standardization.—(A) (1) Obtain oxalated venous blood from a healthy young man with a normal red cell count, and determine carefully the haemoglobin reading (in Gm.) on the instrument to be tested, taking the average of several observations.
(2) Determine the O-combining capacity of the same blood in volumes per cent, and determine the Hb. content in Gm. by dividing this figure by 1.34. If this is not practicable, determine the Hb. with some other instrument which has been so standardized

and is known to be accurate. (3) Determine the correction factor by dividing the correct reading by the reading on the instrument being tested.

For example, if the correct reading is 15 Gm., and the reading on the instrument to be tested is 14 Gm., the standard of the latter is too dark. The correction factor is  $15_{14}$ , or 1.07. If the correct reading is 15 Gm. and the reading on the instrument is 17 Gm., the standard is too pale; the correction factor is  $15_{17}$ , or 0.88.

(B) If neither procedure is possible: (1) Make a careful red cell count on a specimen of normal oxalated blood. It is preferable to use pooled blood from several different individuals. The count must be at least 5.0 million. (2) Dilute the suspension with sufficient physiological salt solution to bring the count just to 5.0 million. Assume that this contains just 14.5 Gm. Hb. per 100 cc. (3) Carefully determine the Hb. of this adjusted blood with the instrument to be tested. (4) The factor is determined by dividing 14.5 by the reading. While this procedure does not give research accuracy, it avoids any error of clinical significance.

The minimum probable error, if the instrument has been accurately standardized, is about 2%, and in actual practice is nearer 5%.

Newcomer's Method.—A colored glass disc is substituted for the acid haematin suspension. This is inserted in one of the light paths of a Duboscq colorimeter under one of the cups which is filled with water. In the other cup put 5 cc. of N/10 HCl, and with a Sahli haemoglobinometer pipette add just 20 cmm. of blood. Rinse, mix well, and let stand from 10 to 60 minutes or longer. Then put the cup in the colorimeter and adjust until the colors match. The Hb in Gm. per 100 cc. is calculated by dividing the appropriate figure in the following table by the colorimetric reading. Precise results can be obtained if the two fields are equally illuminated. It is well to reverse the position of the blood and the standard, take a second reading, and average the two.

Newcomer's Table for Use with the Standard Disc To Obtain Grams Haemoglobin per 100 cc. Blood Divide the Appropriate Figure in the Table by the Colorimetric Reading

Minutes since		Thickness of the colored glass in millimeters									
dilution	0.95	0.96	0.97	0.98	0.99	1.00	1.01	1.02	1.03	1.04	1.05
		0 7 4	-6		. 0						
10	94.4	95.4	96.4				100.4	101.4	102.4	103.4	104.4
15	93.1	94.1	95.0	96.0	97.0	98. <b>o</b>	99.0	100.0	101.0	102.0	103.0
20	92.5	93.5	94.5	95.4	96.4	97.4	98.4	99 - 4	100.4	101.3	102.3
30 .	91.8	92.8	93.8	94.8	95.7	96.7	97 - 7	98.6	99.6	100.5	101.5
40	91.6	92.5	93.5	94.5	95.4	96.4	97 - 4	98.3	99.3	100.3	101.2
Final	90.6	91.6	92.5	93.5	94.4	95.4	96.4	97 - 3	98.3	99.2	100.2
		1					1				

Tallqvist's haemoglobin scale has been much used because of its great simplicity, but the error in the method (10% to 20%) is so great that it can not be recommended. If it is used, the following precautions should be observed. The drop of blood should be large enough to make a stain at least 1 cm. in diameter. Readings should be made in diffuse day light, as soon as the spot loses its gloss. They are best made by cutting out the spot and putting it over the color scale plates. The colors of the standard plates

fade if exposed to light. The readings are very inaccurate (too high) in cases of severe anaemia.

#### ENUMERATION OF THE BLOOD CORPUSCIES

Haemacytometer.—We recommend the Levy-Hausser type of counting chamber with a double platform, and with the "improved" Neubauer ruling. The counting chamber is made of a single solid piece of glass, and is carried in a Bakelite holder which can be used conveniently on a microscope stage, and which protects the chamber from scratching and breakage. In the improved Neubauer ruling the central square mm. is

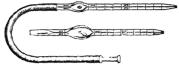


Fig. 54.—Thoma diluting pipettes.



Fig. 55.—Thoma diluting pipette bulb enlarged.

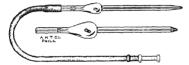


Fig. 56.—Trenner automatic diluting pipettes.



Fig. 57.—Trenner diluting pipette bulb enlarged.



FIG. 58.—Closure for blood diluting pipettes showing (1) Closure in position on rubber tubing, (2) filled pipette closed with finger tip, and (3) rubber tubing sharply kinked prior to rotating tip into position in closure.

FIG. 59.—Closure for blood diluting pipettes showing pipette tip in position in closure. Pipette is now automatically sealed.

divided, as in previous types, into 400 smallest squares. The latter are arranged in 25 groups of 16 squares each by making each fifth vertical and horizontal line a double line, or in the most recent model, a triple line. For diluting the blood the ordinary Thoma diluting pipette is satisfactory. For those who have difficulty in drawing the blood exactly to the mark on the stem of the Thoma pipette, the Trenner diluting pipette is recommended. The hard-rubber closure illustrated is convenient to prevent leakage



Fig. 60.-Levy counting chamber.



Fig. 61.—Levy-Hausser counting chamber.

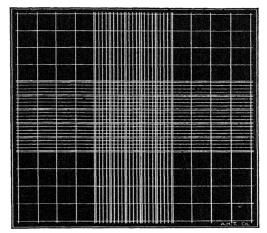


Fig. 62.—Entire area of improved Neubauer ruling showing split boundary lines (400 small squares available for counting.)



FIG. 63.—Group of 16 smallest squares of improved Neubauer ruling showing split boundary lines. Entire group visible simultaneously with 4 mm. objective.

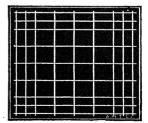


FIG. 64.—Group of 16 smallest squares of original Neubauer ruling showing boundary by fifth squares with extra line in middle of each. The border squares necessitate readjustment of focus.

and facilitate handling of the pipette while it is being shaken. It is desirable to use apparatus which has been certified by the Bureau of Standards.

Enumeration of the Red Corpuscles. Procedure.—The tip of the diluting pipette for red cells, carrying the graduation for on the stem above the bulb, is applied to a fairly large drop of blood; and with the pipette in a horizontal position, blood is drawn by gentle suction just to the 0.5 mark (or to the 1.0 mark if there is marked anaemia). If the column of blood goes slightly past the mark, it may be brought back to the line by gently tapping the tip of the pipette on the finger or on a piece of filter paper. If the Trenner pipette is used, the blood is similarly drawn in until the stem is nearly filled. It is then allowed to flow in by capillary attraction (without suction) until the bore is filled, when it automatically stops. Ouickly wipe off any blood on the outside of the pipette, insert the tip in the diluting fluid, and fill the bulb of the pipette by gentle suction until the fluid reaches the 101 mark on the stem above the bulb. To avoid air bubbles hold the pipette vertically, twirl the pipette between the fingers while it is being filled, and, if the glass bead sticks to the glass, dislodge it by tapping with the finger. At once close the tip of the pipette with the finger or rubber closure, and shake a few seconds to ensure mixing. Up to this point, if capillary blood is being used, one must work rapidly to prevent clotting. The pipette may now be set aside for a few hours before completing the count.

The counting chamber is placed on a level surface, dust is brushed off with a camel's hair brush, and the cover slip is applied. Both must be perfectly clean. If a certified cover slip is used, it must be applied with the Bureau of Standards mark uppermost. Shake the pipette for about 2 minutes, holding the pipette horizontally, and shaking at right angles to its long axis. Blow out two or three drops, and apply the tip of the pipette to the top of the ruled platform of the counting chamber at the edge of the cover slip. Blowing very gently if necessary, allow enough of the diluted blood to flow out to cover the platform completely but not to overflow into the moat. Air bubbles must be avoided. Then similarly cover the other platform. Inspect with the low power of the microscope. If the cells are not evenly distributed, or if the fluid overflows into the moat, clean up the chamber, reshake the pipette, and repeat the procedure until a satisfactory distribution is obtained. Let the chamber stand a few minutes until the cells have settled.

With the high-power dry lens count the red cells in 5 of the groups of 16 smallest squares. To get the most representative distribution it is best to count the four corner groups and the central group. A mechanical counter greatly facilitates the counting. To avoid counting the same cell twice, cells which touch a line are counted if they touch the left hand or upper line of a square, and are not counted if they touch the right hand or lower line, regardless of where the bulk of the cell may lie. We prefer this method, even with the newer types of ruling, and regard a cell as "touching" a line if it is in contact with any part of the double or triple line. Any white cells which are present are also counted. If the leukocyte count is significantly high it may later be subtracted from the total count. Then count the cells on the second platform and take the average of the two counts. They should not differ by more than 20 or at most 30 cells.

Calculation.—The cells have been counted in 5 groups of 16 smallest squares or in 80 of the 400 squares, that is in  $\frac{1}{16}$  of the sq. mm. The depth of the chamber is  $\frac{1}{16}$  mm. The dilution of the blood is (usually) 1 to 200. The total red cell count per cmm. is therefore obtained by multiplying the number of cells counted (average of the two fields) by 10,000 (5 by 10 by 200).

The probable error, with reasonable care and average experience, is about 4%, or 200,000 cells in a total count of 5,000,000. The expert with adequate care can reduce this to about 1%.

Diluting solutions.—Hayem's solution is recommended. Sodium chloride, 1.0 Gm.; sodium sulphate, 5.0 Gm.; mercuric chloride, 0.5 Gm.; water, 200 cc. Filter occasionally.

Toisson's solution is satisfactory. Sodium chloride, 1.0 Gm.; sodium sulphate, 8.0 Gm.; glycerin, 30 cc.; water, 160 cc.

In an emergency 1% sodium chloride solution may be used, but it is less satisfactory. Enumeration of the White Corpuscles.—Using the Thoma (or Trenner) diluting pipette for white corpuscles with the graduation 11 on the stem above the bulb, draw up blood to the 0.5 mark, (to 1.0 if there is a leukopenia) and then draw diluting fluid to the 11 mark, just as directed for counting red cells. As diluting fluid we prefer Türk's solution: glacial acetic acid 1.0 cc.; 1% aqueous gentian violet solution 1 cc.; water 100 cc. Filter frequently to remove yeasts which may grow in the solution.

Shake the pipette and cover the two platforms of the counting chamber with diluted blood, as in counting red cells. Make sure that the cells are evenly distributed by inspecting the entire ruled area under the low power objective. Count the total number of leukocytes on 5 of the 9 sq. mm. areas on each of the two platforms. With some experience this can be done with the low-power objective, although beginners may mistake yeast cells or small pieces of dirt for leukocytes, unless the high dry objective is used.

Calculation.—The total area counted is 10 sq. mm. The depth of the chamber is 10 mm. The cells from 1 cmm. of diluted blood have therefore been counted. The total leukocyte count per cmm. is obtained by multiplying the total number of cells counted on both platforms by the dilution of the blood, usually 20. If the leukocyte count is very high, as in leukaemia, the blood should be diluted 1 to 100 or 1 to 200 in a red cell pipette with Türk's diluting fluid; or the cells may be counted in all or a portion of the central sq. mm., as in counting red cells. The calculation must be altered accordingly. Nucleated red cells, if present, are included in the count. With due care the error should not exceed 5%.

Enumeration of the Blood Platelets.—Many methods have been suggested, none of which are entirely satisfactory.

A. Direct method.—(1) Moisten the inside of a Thoma diluting pipette for red corpuscles with diluting fluid by aspirating a little fluid into the pipette, and expel the fluid. (2) Fill the stem of the pipette to the 1.0 mark from a freely flowing drop of blood. (3) Fill the pipette with diluting fluid. (4) Shake at once, and mount a drop on each platform of the counting chamber. (5) Place the counting chamber in a moist chamber on a level surface, and let stand 15 minutes, until the platelets have settled. (6) With the high-power dry objective count the number of platelets in 5 groups of 16 smallest squares in each platform. (7) Multiply the average count of the two fields by 5000.

As diluting fluid one may use:

- 1. Sodium citrate 3.5% solution.
- 2. Wright and Kinnicutt's solution: Of a 1-300 solution of brilliant cresyl blue, 2 parts; of a 1-1400 solution of potassium cyanide, 3 parts.
- 3. Brilliant cresyl blue 0.2 Gm.; sodium citrate 1.1 Gm.; sodium chloride 0.55 Gm.; water 100 cc.; formalin 0.2 cc. Let stand over night, and filter frequently. The solution keeps well.

#### 4. Howell and Donahue recommend:

Sodium chloride, 2% solution,	50 cc.
K <sub>2</sub> HPO <sub>4</sub> (11.876 Gm. per liter)	40 cc.
KH <sub>2</sub> PO <sub>4</sub> (9.078 Gm. per liter)	10 cc.
Heparin	60 mg.

This should be freshly prepared or kept in the ice box. Before use filter, boil, cool, and centrifugalize at high speed to remove bacteria and other foreign particles which seriously interfere with the accuracy of the count.

Counts obtained by these methods are usually lower (200,000 to 400,000 in normal blood) than by the following method.

Indirect method.—(1) Put two drops of the preceding (No. 3) diluting fluid in a small watch glass coated with paraffin, or in a small cup-shaped cavity dug out of a block of paraffin. (2) Clean the finger as usual, and coat with a thin film of petrolatum (3) Puncture through the petrolatum deeply enough to get a freely flowing drop. (4) With an ordinary platinum loop or toothpick filmed with paraffin transfer a loop of blood to the diluting fluid (about 1 part of blood to 5 of fluid), and mix. (5) Transfer a loop of the mixture to a slide and apply a cover slip (both perfectly clean). (6) Rim with petrolatum, and let stand 15 minutes. (7) Make a red cell count in the usual way. (8) Using the oil-immersion lens, count the number of red cells and platelets in each of a series of microscopic fields until 1000 red cells have been counted. (9) Calculation: The platelet count per cmm. is calculated by multiplying the red cell count of the patient by the number of platelets actually enumerated, and dividing by 1000. By

this method the number of platelets in normal blood is from 300,000 to 800,000.

For practical purposes anyone with a little experience can estimate the number of platelets with sufficient accuracy by inspecting a well made cover slip (stained) film (normally there is one platelet to each 7 to 15 red cells). This is always desirable as a check on the accuracy of a count.

#### VOLUME OF THE RED CORPUSCLES

We recommend the haematocrit tube of Wintrobe. By means of a rubber-bulb pipette with a long, slender tip the tube is filled to the 10.0 cm. mark with well-mixed oxalated blood. To avoid air bubbles, the tip of the pipette should be inserted to the bottom of the tube, and then be gradually withdrawn as blood is expelled into the tube. The sedimentation rate may first be obtained. The tube is then centrifugalized in an ordinary holder until the cells are sedimented to a constant volume. A good electric centrifuge is required, capable of a speed of at least 2500 revolutions per minute. The time required is usually 20 to 30 minutes, but must be determined for the centrifuge. The chief source of error is

inadequate centrifugalization, which gives too high a reading. After packing of the cells is complete, the volume of red cells in per cent is read directly from the graduation at the top of the column of red cells. This must be corrected for shrinkage of the cells if potassium oxalate is used. Multiply by 1.04 if 2 mg. of oxalate per cc. of blood was used.



Fig. 65.— Van Allen haematocrit tube.

Van Allen's haematocrit tube may be used to determine the cell volume with capillary blood. Blood is drawn by suction just to the 100 mark. The anticoagulant, an isotonic (1.3%) solution of sodium oxalate, is then drawn up into the tube until the bulb is about ½ filled. The exact amount is immaterial. The tube is then sealed by drawing a stout rubber band over both ends of the tube, or preferably by one of the spring clips furnished with the instrument. It is then centrifugalized until the cells are packed to a constant volume, and the graduation at the top of the cell column gives the reading directly in per cent. No correction for shrinkage is necessary. It is less satisfactory than the Wintrobe tube, if venous blood is available.

# PREPARATION OF BLOOD FILMS

Films may be made on slides, or on cover slips. For differential leukocyte counts cover slips are much to be preferred, as the leukocytes are more evenly distributed. In films spread on slides the large cells tend to accumulate at the margins and end of the film, whereas the lymphocytes are largely scattered through the middle of the film. Slides are satisfactory for the examination of red cells, for the study of individual leukocytes, and for search for parasites. They are preferable for thick film preparations.

Cleaning Glass Ware.—Cover slips should be 22 mm. square, No. 1 or No. 2, of the best grade obtainable. Cover slips and slides used for making blood films must be absolutely clean and free from grease. To clean cover slips we prefer to drop the slips individually into sulphuric acid-potassium bichromate cleaning solution and let them remain in it 24 hours or longer. The slips are then removed, rinsed individually in running water, then in distilled water, and dropped into 95% alcohol. To dry them conveniently, secure a thin smooth piece of board about 8 inches square, pad it well and wrap tightly with a clean linen towel. With cover slip forceps remove the slips from the alcohol, spread them singly on the padded board, and dry by rubbing over them gently with a handkerchief, (first scrubbing the hands to get them free from grease). Pick up the slips with forceps and store them in a dust-proof box. Never handle with the fingers glass ware that is to be used for blood films. Some prefer to polish the slips with jewelers' rouge, but we have not found this necessary.

Slides may be cleaned in the same way. They may be cleaned by scrubbing with soap and water, rinsing thoroughly, drying, and passing through the flame of a bunsen burner to remove the last trace of grease. More satisfactory results can be obtained by using Bon Ami. Rub up some with the wet finger, rub the slide with the lather until there is a friction squeak, let dry, and polish with a clean dry cloth. This is better than soap and water, alcohol, ether, and flaming combined. The advantage of using such substances as jewelers' rouge, Bon Ami, and strong acids is that they not only remove grease, but probably slightly etch the surface of the glass, and facilitate even spreading of the film.

Cover Slip Films.—With forceps remove several cover slips from the stock box, and brush off any lint or dust with a clean camel's hair brush. Touch a small freely-flowing drop of blood about 2 to 3 mm. in diameter with the center of a clean cover slip, taking care not to let the slip touch the skin. Immediately lower this onto a second cover slip, allowing the weight of the cover slip to spread the drop of blood. No pressure is permissible. When the spreading of the drop begins to slow down, grasp the upper cover slip again, and pull it away from the other in such a way that their plane surfaces are perfectly parallel throughout the manipulation. Any tilting of the upper cover slip away from the lower will ruin the preparation. The cover slips, during the manipula-

tion, may be handled with forceps or with the lingers, as the operator prefers. The films are dried in air, and must be protected from dust and insects.

Films on Slides.—Touch a freely flowing drop of blood about 3 or 4 mm. in diameter with a clean glass slide at a spot about ½ an inch from the end of the slide. Put the slide on the table, and lower the end of a second "spreader" slide onto the first slide, slightly in advance of the drop, so as to make an angle of about 30 degrees with the first slide. Draw the spreader back until the edge touches the drop. As soon as the

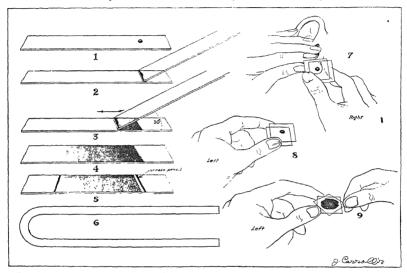


FIG. 66.—1, 2, 3, 4. Making blood smears on slide. 5. Smear ready for staining—grease marks prevent Wright stain from running over slide. 6. U-shaped glass tubing to hold slide in staining. 7. Right hand holding two cover glasses. One cover glass is being touched to drop of blood from ear. 8. Cover glasses transferred to left hand in preparing to place one cover glass on another and spread film. 9. Separating cover glasses by sliding one from the other.

blood runs out along the line of contact, advance the spreader slide with a quick, even sweep to the other end of the slide, so that the blood is pulled (not pushed) along behind the edge of the advancing spreader (see Fig. 66). It is preferable to narrow the spreader slide by filing off a triangular piece about  $\frac{3}{2}$  inch wide at one corner. This makes the film narrower than the slide and facilitates examination of the cells along the margin of the film. By increasing the angle between the two slides thicker films may be obtained and vice versa. In place of the slide a strip of cigarette paper may be used for a spreader, and gives better results in unskilled hands.

#### STAINING METHODS

For routine purposes some modification of the Romanowsky polychrome methylene blue stain is almost universally used. Of these we regard Giemsa's stain as the best. However, the stain is expensive, and considerable time is required for staining the films. For these reasons a simpler type of stain is usually employed. We recommend Wright's stain, which is most generally used in this country, or Wilson's stain, which gives practically identical preparations. Wilson's stain is much better for the demonstration of blood platelets. We recommend the purchase of these stains in solution ready for use, or in the form of dried powder or tablets, to be dissolved in methyl alcohol when needed. The methyl alcohol must be of the highest purity, anhydrous, and free from acetone. The stains may be prepared as follows.

Wright's Stain.—Add I Gm. methylene blue ("medicinally pure," or rectified for Wright's stain) to 100 cc. of a 0.5% solution of sodium carbonate in distilled water, and heat for one hour in an Arnold sterilizer, in flasks large enough so that the layer of fluid is not over 2½ inches deep. Cool, filter, and add about 500 cc. of a 1-1000 solution of yellowish water-soluble eosin. Add the eosin solution slowly, stirring constantly, until the blue color is lost, the mixture becomes purple with a yellow, metallic lustre on the surface, and a finely granular, black precipitate forms. Collect the precipitate on a filter paper, and dry thoroughly in the incubator at 37°C. Dissolve 0.1 Gm. of the powder in 60 cc. of pure methyl alcohol, first grinding up the powder in a mortar with a small amount of the alcohol. Filter before use. Keep in the dark, in tightly stoppered bottles, well protected from moisture and from acid fumes. Fresh solutions are often alkaline, but tend to become acid on standing. If this is not adequately corrected by using buffered diluting fluid, it can sometimes be done by mixing old and new solutions, or by adding minute traces of glacial acetic acid or solid sodium hydroxide.

Wilson's Stain.—Dissolve 2.0 Gm. silver nitrate in 50 cc. of water, and add a 2% to 5% solution of sodium hydroxide until the silver oxide is completely precipitated. Wash the heavy, black precipitate several times by pouring distilled water into the flask, shaking, and decanting until all the alkali is removed. To the moist silver oxide add a solution containing 2 Gm. of methylene blue in 200 cc. of 0.5% solution of sodium carbonate in distilled water.

Boil this silver oxide-methylene blue mixture gently in a rather deep porcelain dish for 30 minutes, stirring occasionally. Pour off and preserve about one third of the contents of the dish in a 200 cc. graduated cylinder. Add to the contents of the dish an amount of boiling distilled water equal to that of the fluid poured into the cylinder, and boil for 30 minutes. Again pour off into the same cylinder one third of the contents of the dish. Boil for 30 minutes more, without adding additional water. Add the contents of the dish to the cylinder and make up to 200 cc. with distilled water. Filter into a 500 cc. beaker and immediately add a solution containing 1 Gm. of yellow water-soluble eosin dissolved in 200 cc. of distilled water. Let the mixture stand for about 30 minutes and collect the precipitate on a hard filter paper. Dry in the incubator, or in a drying oven at 60°C. The yield of precipitate is about 1.7 Gm. Dissolve 0.2 Gm. in 50 cc. of pure methyl alcohol and store as in the case of Wright's stain.

Technique of Staining.—For the best results the (air dried) films should be stained within 24 hours. Cover slips are most conveniently handled by using a cover slip forceps, such as the Stewart type, which holds the cover in a perfectly horizontal position. For slides see Fig. 66. (1) Cover the dry film with the alcoholic solution of the stain for 1 minute. This fixes the film. (2) Add rapidly, best with a rubber-bulb pipette, an equal quantity of diluent, and allow to stain 5 minutes, (or longer if neces-

sary, until a metallic scum forms on the surface). (3) Wash by flooding the film with distilled water or diluent, until it has a pinkish tinge. (4) Dry in air by tilting the cover slip on one edge so that the water will drain off, or blot carefully with fine blotting paper.

Trouble from the deposition of a precipitate on the film may be avoided by placing the cover slip with the film down in a small shallow watch glass, and adding with a pipette enough of the undiluted stain just to float the cover slip. After I minute add an equal volume of diluent, taking care to keep the cover slip floating on the diluted stain. After 5 minutes remove with forceps, wash, and dry as above.

The dried film may then be mounted in immersion oil (cedar oil) for examination, or, if it is to be preserved, in special neutral Canada balsam (which may be purchased from reliable dealers). To ensure a neutral mounting medium make a relatively dilute, thin solution of balsam damar in xylol, add powdered calcium oxide, shake well, and let stand several weeks until the mixture clears by sedimentation.

As diluent one may use CO<sub>2</sub>-free distilled water, or (if the stain is alkaline) the following buffered phosphate solution. Recrystallized mono-basic potassium phosphate 6.63 Gm.; anhydrous dibasic sodium phosphate 2.56 Gm. (both of the highest purity); distilled water 1 liter. This should have a pH of 6.4. To preserve, add 1 cc. of chloroform to the stock bottle.

If these reagents are not available and the distilled water is too acid, the following may be tried. (1) Boil the water to expel CO<sub>2</sub>, and cool. Test by adding to a few cc. in a small test tube a bit of haematoxylin. If the color does not turn blue in from 1 to 5 minutes, the water is still too acid. Correct by adding traces of 1% sodium carbonate solution until it becomes blue. (2) To 5 cc. of distilled water which has been boiled and cooled, add a small drop of a 1% aqueous solution of neutral red. If the color turns pink (acid), add with a glass rod successive traces (not drops) of 1% sodium carbonate solution, until it turns a pinkish yellow (neutral). This may be used. If it turns clear yellow it is too alkaline. Discard, and start again with fresh water.

If distilled water is not obtainable, rain water collected in the open is the best substitute.

Giemsa's Stain.—Dissolve 0.3 Gm. of Azur II eosin and 0.08 Gm. of Azur II (preferably Grübler's) in 25 cc. of pure anhydrous glycerin at 60°C. Add 25 cc. of absolute methyl alcohol at the same temperature. Allow the solution to stand over night and filter. Immediately before use dilute one part of the stain with 10 to 15 parts of distilled water or buffered solution. If intense staining is desired, dilute with 1 to 1000 solution of potassium carbonate.

Procedure.—Fix the film in absolute methyl alcohol for 1 to 5 minutes. Dry. Cover the cover slip with diluted stain, or, preferably, float it on the diluted stain, and leave for 15 to 30 minutes. Wash in distilled water until the film has a pinkish tinge, dry, and mount.

The alkaline diluent is used to demonstrate the coarse stippling in the red cells in malignant tertian malaria, and to stain spirochaetes. To demonstrate *Treponema pallidum* stain for 2 to 12 hours.

The panoptic stain (or "combined Giemsa") is somewhat superior to the simple Giemsa stain for blood films. The air-dried film is first stained with Jenner's stain (identical with the May-Grünwald stain), or with Wright's stain, as above outlined. The film is washed and without further fixation is counterstained with diluted Giemsa's stain.

Haematoxylin and eosin are useful if good preparations of the preceding stains are not available, and is preferred by many for Arneth counts. Films are best fixed by heat, although alcohol may be used.

Heat fixation.—(1) If a drying oven is available, put the films in the oven, heat gradually to  $150^{\circ}$ C., and allow to cool gradually in the oven.

- (2) Put the films (preferably more than 24 hours old) face down on a copper bar, along with a crystal of urea. Heat gradually over a small flame until the crystal melts  $(135^{\circ}\text{C.})$ , and let the bar cool. Todd advised using a tartaric acid crystal, which melts at about  $168^{\circ}\text{C.}$ , and removing the films when the crystal melts. If fresh films are to be fixed, leave the films 2 minutes after the flame has been removed. The flame should be so adjusted that 5 to 7 minutes are required to melt the crystal.
- (3) Pour absolute alcohol over the film, quickly drain off the excess, and ignite the remaining film of alcohol.

Procedure.—(1) Fix the film, preferably by heat. (2) Stain with any good haematoxylin solution, such as Delafield's, (or Meyer's haemalum) for 5 to 15 minutes. (3) Wash in tap water for 2 to 5 minutes to develop the haematoxylin color. (4) Stain either with a 1 to 1000 aqueous eosin solution, or with a 0.5% eosin solution in 70% alcohol for 15 to 30 seconds. (5) Wash and examine.

This method stains nuclei and eosinophile granules sharply, but it does not stain neutrophilic granules, platelets, malarial parasites, or basophilic cytoplasm in red cells.

Kingsley's stain.—Solution 1.—Methylene blue, U. S. P., med. 88%, 0.065 Gm.; Methylene azure A 80%, 0.010 Gm.; glycerin, C.P., 5 cc.; methyl alcohol, C.P., 5 cc.; distilled water, 25 cc.; buffer, pH 6.9, 15 cc.

Solution 2.—Methylene violet (Bernthsen) 85%, 0.013 Gm.; Eosin, yellow, water soluble, 92%, 0.045 Gm.; glycerin, C.P., 5 cc.; methyl alcohol, C.P., 10 cc.; acetone, C.P., 35 cc.

The dyes must be weighed accurately. These solutions are permanent if stored in clean glass bottles with tight, ground glass stoppers.

Buffer solution.—(1) Dissolve 9.078 Gm. KH<sub>2</sub>PO<sub>4</sub> in 1 liter of water. (2) Dissolve 11.876 Gm. of Na<sub>2</sub>HPO<sub>4.2</sub>H<sub>2</sub>O in 1 liter of water. Add a thymol crystal to each. This preparation of the disodium phosphate may be prepared from the recrystallized salt (which contains 12 molecules of water of crystallization) by exposing the latter to the air for two weeks. A pH of 6.9 is obtained by adding 40 cc. of (1) to 60 cc. of (2).

The working solution is prepared by mixing equal parts of Solution 1 and Solution 2. This deteriorates slowly, but may be used for several months.

Procedure.—Fix the air-dried blood film with absolute methyl alcohol for 1 minute. Dry. Flood with working staining solution and stain for 5 to 7 minutes, or until a uniform metallic scum forms on the surface of the fluid. Wash with a stream of distilled water, dry, and mount.

The advantages of this stain over the Romanowsky stains are that its composition is constant, and solutions of uniform staining properties can be prepared. The solutions are permanent. The technique is simple, the only variable being the time of staining. The appearance of the cells is similar to that obtained with a good preparation of Wright's stain.

Peroxidase Stain.—Good pasture's stain is simple and gives excellent preparations, but some experience with the stain is necessary to get satisfactory results consistently. Formula: Sodium nitroprusside, 0.05 Gm.; benzidine (C.P.) Harmer, 0.05 Gm.; basic fuchsin, 0.10 Gm.; alcohol, 95%, 100 cc. Dissolve the nitroprusside in about 1 cc. of

water in a mortar, and add the alcohol. Then add the other ingredients. The stain (without peroxide) should keep for several months. A weak stain is usually due to deterioration of the benzidine, or a poor preparation. It may sometimes be revived by adding a little additional benzidine.

Procedure.—Use air-dried films less than 24 hours old. (1) Cover film with the stain and let stand 1 minute to fix. (2) Add an equal volume of 1 to 200 dilution of hydrogen peroxide, and stain 5 minutes. (3) Wash in water, dry and mount. They may be counterstained with Wright's stain if desired.

Sato and Sekiya's stain is preferred by some workers. Solutions: (1) Copper sulphate 0.5 Gm. in 100 cc. of water. (2) Grind 0.02 Gm. of benzidine in a few drops of water, and dilute to 200 cc. Filter. Add 0.25 cc. of 3% hydrogen peroxide. Store in the dark in a brown bottle. (3) 1% aqueous solution of safranin.

Procedure.—(1) Cover film with copper sulphate solution for 20 seconds. (2) Drain and apply the benzidine solution for 8 minutes. (3) Drain and stain with safranin solution for 2 minutes. (4) Wash, dry and mount.

Make sure that the solutions used stain the granules in normal blood before attempting to stain pathological blood.

The granules of the granular leukocytes are stained a deep blue (or brownish in thick overstained films). Myelocytes and premyelocytes contain similar granules. Monocytes contain a smaller number of fine, bluish granules. Lymphocytes never contain granules. The method is, therefore, useful in differentiating lymphocytes from early myelocytes in acute leukaemia. The fact that myeloblasts in the strict sense do not show granules, despite earlier statements to the contrary, to some extent restricts the conclusions that can be drawn from the method.

Indophenol Blue Synthesis (Schultze).—This test yields practically the same information as the peroxidase stain, and is preferred by some workers.

(1) Fix films for 15 to 20 minutes in equal parts of 40% formaldehyde and absolute alcohol. (2) Stain 3 minutes in a mixture of one part of Solution I and four parts of Solution II. (3) Films may be counterstained for 3 minutes in Giemsa's stain. (4) Mount in water.

Solution I.  $\alpha$ -naphthol 1.0 Gm.; physiological sodium chloride solution 100 cc.; N/10 potassium hydroxide 1 cc. Filter.

Solution II. Paradimethyl-paraphenylene-diamine r Gm.; physiological sodium chloride solution 100 cc.

The granules of the myeloid leukocytes and monocytes stain intensely blue. In many cases of myelogenous leukaemia cells classed as myeloblasts take the blue stain. Not all myeloblasts do so, however ("Oxydasenschwund" of Jagic). Lymphocytes never stain.

Differential Count.—The stained film is inspected, and if the cells are evenly distributed, well stained, and not smudged, it is examined under the oil-immersion lens with a mechanical stage. If the film is on a cover slip, it is desirable to move the slide back and forth from one margin of the film to the other, in the direction in which the films were pulled apart. Every consecutive cell should be identified and recorded until at least 250 cells have been counted. If the count is abnormal a second 250 should be counted on a second cover slip. If the film is made on a slide, the count should be made by moving back and forth at right angles to the margin of the film from the edge of the film to a point several microscopic fields in from the margin. In this way the uneven distribution of leukocytes in such films is partly compensated. As a minimum, count

50 cells at four (better five) different parts of the film. (Schilling's four field meander method.)

Separate headings should be made for each of the types of normal leukocytes and for all the immature types which may be present. The neutrophiles should be subdivided into cells with completely segmented nuclei, and cells with incompletely segmented, band-shaped nuclei of mature and immature type. Nucleated red cells should be recorded but not included in the 250 cells counted. Occasional smudged cells must be included, and placed in the proper group if they can be identified, or recorded as smudges if identification is not possible. Films of normal blood containing many smudges are not fit for a count, but in some pathological conditions in which large, fragile cells are present, it may be impossible to avoid smudges.

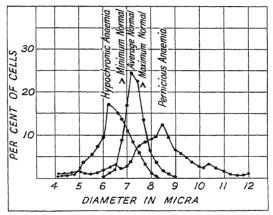


Fig. 67.—Price-Jones curves of normal blood, and of cases of hypochromic and pernicious anaemia. (Combined and modified from Price-Jones.)

Determination of the Diameter of the Red Corpuscles. Price-Jones Curve.—By means of a haemacytometer chamber or some accurate stage micrometer, calibrate an ocular micrometer for use with the oil lens. It greatly facilitates the measurements if, by altering the length of the draw tube, 50 points on the micrometer scale can be made to coincide with the width of one of the smallest aquares of the haemacytometer  $(50\mu)$ . Then measure precisely the diameter of at least 200 red cells (preferably 500) in a thin film stained by Wright's stain. It has been shown that the average diameter in well made films is the same as in fresh preparations. Move the slide with a mechanical stage from right to left, with the long axis of the micrometer graduations parallel to that of the slide, and measure to the nearest  $0.5\mu$  the horizontal diameter of every round (not distorted) cell passing across the center of the field. Many English observers measure to the nearest  $0.25\mu$ , but this is scarcely possible without special projection apparatus. Plot these figures on a chart, as in Fig. 67. It is useful also to plot the figures of normal blood for comparison. Such a graph gives a precise estimate of the degree of microcytosis or macrocytosis present, and also of the anisocytosis.

Normal blood shows a curve with a high peak and a narrow base. According to Price-Jones the mean figure for the peak in normal blood is  $7.2\mu$ , with a possible range from 6.7 to  $7.7\mu$ . Many observers in this country have reported figures about  $0.4\mu$  higher. Each observer must establish his own normal standard by counts on normal blood. Two thirds of the cells fall within  $0.5\mu$  of the peak value. In anaemias the peak is lower and the base broader. The degree to which the base of the curve is spread out is a measure of the anisocytosis. This may be expressed numerically by calculating the coefficient of variation, which is 5.8 for normal blood, and may reach 13 or more in severe anaemias. In pernicious anaemia and other macrocytic anaemias the peak is at a point above  $7.7\mu$ , while in most "secondary" anaemias, in chlorosis and in idiopathic hypochromic anaemia it is below  $6.7\mu$ . The procedure is tedious and impracticable for routine clinical purposes, but in selected cases it gives information that can not be obtained in any other way.

The average diameter of the red cells in a film can be determined simply and quickly, but with less precision, by means of the *halometer* of Pijper (Brit. M. J., 1929, 1, 635). This does not determine the degree of anisocytosis, however, and if anisocytosis is marked, it is difficult to obtain precise readings.

Thick films are of the greatest practical assistance in searching for malarial parasites, particularly when they are present only in small numbers in the peripheral circulation, for trypanosomes, spirochaetes of relapsing fever, and filarial embryos. The method as described by Benavides (1934) has given very satisfactory results at the U. S. Naval Medical School, and is recommended. Experience is necessary in staining and interpreting thick films.

A large drop of blood is spread on a slide with a tooth pick, or by tilting the slide, so that the blood is spread over an area ½ inch in diameter. This is thoroughly dried in air. It ordinarily requires 1 to 2 hours at room temperature, or 30 minutes in the incubator. The film must be protected from insects while drying.

A modified Giemsa stain is used. All utensils must be chemically clean, and the reagents of the highest grade. Put 2.4 Gm. of Grübler's Azur II-eosin in an Erlenmeyer flask, add 200 cc. of anhydrous glycerin, stopper, and heat in a water bath at 60°C. for 30 minutes, shaking occasionally. Then add 200 cc. of anhydrous methyl alcohol, mix, and return to the water bath, shaking once or twice. Place in an incubator over night. Filter, and store the stock stain in tightly stoppered bottles in the dark.

For use, add 1 cc. of the stain to 30 cc. of distilled water (1 or 2 drops to 1 cc.) having a pH of 7.0 to 7.2, or fresh rain water. Mix, and put in a staining jar. Immerse the dried film, without fixation or preliminary laking, in the diluted stain in a vertical position, and leave an hour and a half. Rinse by dipping gently in two changes of water. Dry thoroughly, and mount in oil or balsam. The chromatin of the parasites takes a more brilliant red color than with the ordinary Giemsa or Wright stain.

Basophilic aggregation test (McCord et al., 1924).—This is useful in cases of suspected lead poisoning, since it concentrates the cells to some extent and makes the basophilic material more readily visible. (1) Make films about three times as thick as for a differential count, and dry thoroughly as for thick films. (2) Without fixation stain for ten minutes with Manson's methylene blue (2% methylene blue and 5% borax in distilled water). (3) Rinse off stain by dipping gently into distilled water several times and dry thoroughly. (4) Examine under oil and count the number of clumps of the small dark blue granules in a large number of fields.

### SUPRAVITAL STAINING METHODS

Reticulocytes.—To demonstrate reticulocytes the red cells must be brought into contact with the stain while fresh and moist. Brilliant cresyl blue is usually employed. We prefer Grübler's Azur II.

- (1) Clean cover slips are coated with a thin film of cresyl blue by dipping them into or flooding them with a 0.5% alcoholic solution of the stain, and allowing them to dry. Blood films are made on these cover slips in the usual way and dried in air. They may then be mounted in balsam and examined, or they may be counterstained with Wright's stain. This method is widely used and gives fairly satisfactory results for routine clinical purposes, but it does not show all the reticulocytes.
- (2) A clean slide is filmed with stain, as described above, and a drop of blood on a clean cover slip is mounted on the slide, rimmed with vaseline, and examined after 30 minutes. This method stains the reticulocytes more heavily and in somewhat larger number. By these methods the count in normal blood is from 0.1% to 0.5%.
- (3) Osgood's method (1934) is more efficient. In a small test tube mix equal volumes of oxalated blood and a 1% solution of cresyl blue in physiological salt solution. Let stand I minute or more. Mix, and make thin films. Dry in air, mount in oil, and examine within 24 hours; or counterstain with Wright's stain to secure permanent preparations. Blood 24 hours old may be used. This method gives counts 2 to 3 times higher than the preceding methods. Osgood's figures for normal blood are 0.5% to 3.8%, averaging I.5%.
- (4) Dissolve I Gm. of *Grübler's Azur II* in 100 cc. of absolute ethyl alcohol. This stock solution seems to work better after standing for about 6 months.

Dilute one part of stock solution with four parts of absolute ethyl alcohol. Dip slides cleaned as in method (2) into this working solution of stain, drain and dry in air. Polish lightly with lens paper. Touch a drop of blood with the center of a clean cover slip, lay the cover slip, drop up, on a flat surface, and gently lower the slide onto it so that the weight of the slide causes even spreading of the drop. Seal with vaseline and examine after ro minutes. This method stains the reticulocytes with maximum intensity, so that even single granulations stand out conspicuously. Counts obtained by this method are much higher than by the usual methods and can not be compared directly with them. The leukocytes are also stained by this procedure, and retain their vitality and motility for a considerable time. The lymphocytes are recognized by a single nucleolus; neutrophiles, by sharp, elongated, finger-like pseudopods; eosinophiles, by a regular, mosaic type of granulation; basophiles, by irregular clumping of the granules and early staining of the nucleus; and monocytes, by blunt, wide pseudopods and occasionally by the ingestion of foreign particles.

This method has been used with satisfaction in the laboratories of the U. S. Naval Medical School.

On a film stained by any of these methods count the total number of red cells and of reticulocytes in a series of oil-immersion fields until 1000 red cells have been counted. To reduce the size of the field to practicable dimensions insert in the eye piece a piece of stiff paper with a square hole about ½ inch in diameter cut in the center.

Leukovytes (Sabin's method).—(1) Perfectly clean slides are brushed free from dust, flamed, and dipped in a dilute alcoholic solution of biological neutral red. This is drained off immediately, and the slides are placed upright on one end and dried in air.

For average blood a 0.04% solution is used. Prepare a stock solution by dissolving 0.1 Gm. of neutral red in 10 cc. of absolute alcohol. For use add 0.4 cc. of this solution to 10 cc. of absolute alcohol.

(2) To 2 cc. of this dilute solution add 3 drops of a saturated solution of Janus green in absolute alcohol. Similarly prepare slides with this solution of the combined stains.

With a clean cover slip mount a drop of fresh blood on one of these slides as in making an ordinary fresh preparation. It must spread evenly without pressure. Rim with petrolatum, and put at once in a warm box or on a thermostatically controlled warm stage, at 37°C. Examine with the oil lens, taking care not to jam the objective down onto the cover slip when focussing.

For most purposes the neutral red alone is preferable, as it is practically non-toxic, and the cells usually remain alive and motile for an hour or more. Janus green stains the mitochondria which are numerous in immature cells, and thus gives additional information, but it is quite toxic. The cells usually lose their motility and die after 15 to 20 minutes.

In neutral red preparations the granules of the *polymor phonuclear neutro philes* appear as uniform, fine, faintly reddish-stained structures. There may be one or two red vacuoles present. The healthy cells show constant active amoeboid motility with streaming of the granules within the cell. They often move across the field with surprising rapidity. Except when dying they are rarely seen rounded up, as in fixed films.

The eosinophiles contain round, coarse granules of uniform size, somewhat more deeply stained than the neutrophilic granules. They also show motility, but are somewhat less active than the neutrophiles.

The basophiles show round granules varying in size and depth of staining, but some are usually more deeply stained than in the preceding types. They show sluggish amoeboid motility.

The granules of the *myelocytes* take the red stain in a similar manner. These cells are not motile, show no streaming of the granules, and contain no vacuoles.

The lymphocytes show a strikingly clear cytoplasm without any granules. They contain from one to several reddish-staining vacuoles which are variable in size and shape, and may increase in size as the preparation stands. With Janus green they typically show a clump of relatively coarse rod-shaped mitochondria opposite the nucleus. The large lymphocytes and many of the small ones show little or no motility, but many of the intermediate forms show sluggish progressive amoeboid motility which is characterized by the fact that the nucleus is usually in the front of the advancing cell. The coarse chromatin masses in the nucleus can easily be seen.

The monocytes show red particles which vary from fine granulations to coarse vacuoles. They usually contain moderately numerous, small vacuoles, coarser and redder than the neutrophilic granules. In the moving cell these show streaming within the cytoplasm, but when the cell rounds up they tend to be arranged in a rosette pattern about the centrosphere in the concavity of the nucleus. The cells often show progressive amoeboid motility which is much more sluggish than that of neutrophiles. They may round up, and show numerous, relatively small pseudopods along their margins, which may be slender and filiform, or blunt and short with irregular margins, quite similar to the irregularities occasionally seen in stained films. They are actively phagocytic. Janus green stains fine mitochondria diffusely scattered through the cytoplasm.

Sabin differentiates the monocytes sharply from the clasmatecytes, or histiocytes, the wandering phagocytic cells of the reticulum, which appear in the blood in certain pathological conditions, and occasionally in small numbers in normal blood. These cells are more actively motile and phagocytic than the monocyte. They show more numerous vacuoles which are often coarser and vary more in size and color than in the case of the monocyte, and show no tendency to assume the rosette pattern.

In the hands of Sabin and her associates this method of staining has been of great value as a research procedure. For ordinary clinical purposes its chief value is in the identification of monocytes, and in their differentiation from myelocytes on the one hand and from lymphocytes on the other. The distinguishing criteria are more definite and decisive than in the case of stained films. It is also of assistance in the recognition of immature cells, rich in mitochondria

Sabin emphasizes that no valid conclusions can be drawn from preparations in which the cells are dead. She regards any staining of the nucleus as indicating a dead or dying cell. Those not skilled in the procedure are advised to depend mainly upon preparations stained with neutral red alone.

#### MISCELLANEOUS EXAMINATIONS

Fragility of the Red Corpuscles (resistance of the red cells to hypotonic salt solution).—(1) In a porcelain evaporating dish put a few grams of pure sodium chloride, and heat over a flame with constant stirring until all moisture has been driven off. Cool in a desiccator. Make a 1% solution by dissolving exactly 1 Gm. in distilled water in a 100 cc. volumetric flask, dilute to volume, and mix.

- (2) In test tube racks set up two series of 15 small test tubes each, using glass which has been acid-cleaned, thoroughly rinsed (the last time in distilled water), and dried. Prepare in each set of tubes a series of dilutions of salt solution, ranging from 0.3% to 0.6%, in steps of 0.02%, with a volume of 2 cc. in each tube. This may be done conveniently by adding to each tube in each set (with a serological pipette) first 1% salt solution beginning with 0.6 cc. in the first tube, and increasing the quantity by 0.04 cc. in each succeeding tube; and, second, distilled water, beginning with 1.40 cc. in the first tube, and diminishing the quantity by 0.04 cc. in each successive tube. In laboratories in which many tests are made it is simpler to prepare each dilution in 100 cc. quantities, and add 2 cc. of the proper dilution to the corresponding tubes when setting up the test. In tightly stoppered bottles they keep for several months.
- (3) To each tube in the first set add one drop of the blood to be examined, and to each tube in the second set one drop of normal (control) blood. The control series with normal blood must not be omitted. One may aspirate 2 cc. of blood from a vein and drop it directly into the tubes from the tip of the needle, without any anticoagulant. This necessitates precise and speedy manipulation to avoid clotting. We prefer to aspirate the blood into a syringe containing  $\frac{1}{10}$  its volume of 3% sodium citrate to prevent clotting (oxalate may be used). Pack the cells in a graduated centrifuge tube, pipette off the supernatant fluid, and suspend in an equal volume of physiological salt solution. With a capillary pipette add one drop of cell suspension to each tube. The syringe (as well as the glass ware) used must be dry or rinsed with physiological salt solution.
- (4) Shake the tubes well and set aside (at room temperature or in the ice box) for two hours or more, until sedimentation is complete.

- (5) NOTE—(1) the concentration of salt in the first tube in each series in which a trace of haemolysis appears (the minimal resistance, normally about 0.44%); and (2) that in the first tube in each series in which haemolysis is complete and there is no red sediment (normally about 0.32%). The presence of a little colorless sediment (stroma) is disregarded.
- (6) Compare the figures for the blood in question with those for the normal control. A clear-cut difference of 0.04% may be regarded as significant.

The resistance is diminished (fragility increased) chiefly in haemolytic jaundice. In most other types of anaemia and in obstructive jaundice the resistance is normal or slightly increased.

Haden (1934) has shown that the diminished resistance is probably not due to any abnormality in the chemical composition of the cell, but to its unusual shape. When red cells are immersed in hypotonic salt solution they absorb water and swell until the osmotic pressure is equalized. The normal red cell, which is a flat disk, can increase markedly in volume by assuming a more spherical shape, before physical rupture of the cell occurs. The cells in haemolytic jaundice are narrower and thicker, are more nearly spherical to start with, and a relatively slight degree of swelling suffices to cause their rupture and haemolysis. There is no constant relation between diminished resistance to hypotonic salt solution and susceptibility to other haemolytic agents.

Sedimentation Rate of the Red Corpuscles.—This is determined by filling a suitable graduated tube with well-mixed oxalated blood, and either observing the time required for the upper level of the column of red cells to fall to some fixed point in the tube, or, preferably, observing the amount of fall during certain definite time intervals. Many different methods, each with its own special tubes, have been employed, of which those of Linzenmeyer, Westergren, and Cutler are the best known. Any one of these gives satisfactory results, but the normal standards are different for each method.

Wintrobe and Landsburg method.—We have found Wintrobe's haematocrit tube satisfactory and very convenient, since with it an accurate determination of the cell volume can subsequently be made with the same specimen of blood. (I) Fill the tube to the 10.0 cm. mark with well-mixed oxalated blood. (See section on Determination of Cell Volume). Blood may be used within 3 or 4 hours after venepuncture; older specimens give too slow a rate. (2) Put the tube in exactly a vertical position in a suitable rack, or in plasticine. It must not be moved or jarred during the period of observation. (3) Read at the end of one hour, and in special cases also after 10, 20, and 30 minutes. (4) Determine the cell volume as previously directed. (5) Correct (approximately) for the accelerating effect of anaemia, if present, by means of the chart, page 313.

Find the horizontal line on the chart corresponding to the observed sedimentation rate at the end of 1 hour, and the vertical line corresponding to the observed cell volume. Follow the curved line nearest the point of intersection of these two lines downward and to the right until this intersects the vertical line which corresponds to the average normal cell volume (42 for women, 47 for men). The marginal reading of the horizontal line nearest this second point of intersection gives the corrected sedimentation rate.

The corrected rate for normal individuals during the first hour is 2 to 9 mm. (rarely over 6 mm.) for males and 2 to 20 mm. (rarely over 15 mm.) for females. If the sedimentation rate for females is corrected for a normal cell volume of 47% (instead of 42%), the normal figures are the same as for males, although there is greater variation among females than among males. Although this correction largely eliminates the effect of

anaemia on the sedimentation rate, the test still can not be regarded as precise. In cases with severe anaemia the corrected figure is often within the normal range, even though some pathological condition is present which usually causes an acceleration of the rate. Both the uncorrected and the corrected figures, therefore, should be recorded in all cases.

Cutter's method is simple and has been extensively employed. Aspirate into a dry, sterile syringe 0.5 cc. of sterile 3% sodium citrate solution. Puncture the vein, and

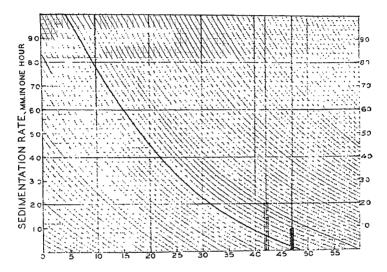


FIG. 68.—Chart for correcting sedimentation rate for variations resulting from differences in the concentration of red corpuscles as measured by volume of packed red cells.

The logarithmic curve on which the chart is based is heavily outlined. The mean normal volume of packed red cells for men (47 cc.) and for women (42 cc.) are also heavily outlined, and the range of normal sedimentation is represented by solid and open columns for each sex, respectively. (Courtesy of Dr. M. M. Wintrobe and J. W. Landsberg, from the American Journal of Medical Sciences.)

aspirate blood to the 5 cc. mark. Draw back the piston, mix the contents of the syringe, remove the needle, and expel the blood into a Cutler sedimentation tube. Put in a rack in a vertical position, and read the upper level of the column of red cells every 5 minutes for an hour. The observations may be plotted on special charts (see Fig. 69). The normal readings at one hour are given as 2 to 8 mm. for men and 2 to 10 mm. for women.

Many different factors influence the sedimentation rate, and the mechanism of the process is not understood. In general, the higher the column of blood, the greater will be the fall (in mm.) in a given interval. The rate varies inversely with the number of

red cells present. It is markedly accelerated in a variety of pathological conditions in which there is an increase in the globulin, and particularly in the fibrinogen of the plasma. Microscopic examination shows that the accelerated sedimentation rate is associated with, and closely parallels, increased rouleaux formation (Fahraeus). The rouleaux are much larger and more compact than in normal blood.

An accelerated sedimentation rate has been observed empirically chiefly in: (1) Anaemias of all types. (2) Normal pregnancy after the

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second or third month, and the puerperium. (3) Acute infections of all types, and active chronic infections, including syphilis and tuberculosis. (4) "Nephrosis." (5) Advanced malignant tumors. (6) Internal haemorrhages. (7) Any condition associated with necrosis or extensive destruction of tissue, including coronary thrombosis. It is not, as a rule, increased

in relatively inactive, chronic focal infections, in benign tumors, in unruptured ectopic pregnancy, nor in purely psychoneurotic states.

It is thus not specific, and like fever or leukocytosis, it is not diagnostic of any particular disease. It is occasionally not accelerated in individuals showing pathological conditions in which this would be expected. An accelerated rate points to the existence of significant organic disease, and indicates the need for thorough investigation. We have found it useful, on this basis, as part of a routine diagnostic study.

In general there is a rough parallelism between the activity of an infection and the degree of acceleration of the rate, particularly the fall during the first 15 to 30 minutes. The relationship in the case of tuberculosis is shown in the preceding chart from Cutler. He regards the sedimentation rate as more valuable than the temperature curve, the pulse rate, or the body weight as an aid in estimating the activity of tuberculosis and in guiding treatment, and advocates the test as a routine procedure in the study of this disease.

Blood Volume.—This may be determined by the method of Keith, Rowntree, and Geraghty. (1) Prepare a 1.5% solution of Grübler's Congo red in distilled water (0.385 Gm. in 25 cc.), filter, and sterilize. (2) Withdraw ro cc. of blood with a dry syringe and put into an oxalate tube (for the standard). (3) Inject the dye solution intravenously through the same needle (avoiding any leakage). The dose in cc. is determined by dividing the body weight in kilograms by 4. (4) Four minutes later (not less than 3 nor more than 6) withdraw 10 cc. of blood from the other arm using a clean syringe and put in an oxalate tube. (5) Centrifugalize both specimens in graduated tubes, until the cells are packed. Read the volume of the cells and plasma in both tubes and take the average. (6) Prepare a standard by adding to 2 cc. of plasma from the first tube 4 cc. of 0.9% sodium chloride solution, and 2 cc. of a 1 to 100 to 100 the 1.5% solution of the dye. (7) To 2 cc. of plasma from the second tube add 6 cc. of 0.9% salt solution. (8) Read in a colorimeter. Calculation:

Plasma volume in cc. = No. cc. of dye solution injected 
$$\times \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 200$$

Blood volume (cc.) =  $\frac{\text{Plasma Volume (cc.)} \times 100}{\text{Plasma volume per cent}}$ 

The result is usually expressed in terms of cc. of blood per kilo of body weight. The average normal figure for adults by this method is 88 cc. per kilo. (72 to 105 cc.). The method is not applicable to patients with "nephrosis" or amyloidosis. (See Congo red test for amyloidosis.)

By the carbon monoxide method the average normal rolume is 56 cc. per kilogram. It is preferable in conditions showing abnormalities in the red cell count, but requires the special equipment and training necessary for precise gas analyses. The reason for the difference in these figures is that the carbon monoxide is all combined with

haemoglobin within the cells, whereas the dye, which is dissolved in the plasma, penetrates into partly collapsed capillaries into which the cells can not enter. Both methods measure only the blood which is in active circulation and not that which is in the spleen pulp and other stagnant areas.

The blood volume is markedly increased in erythraemia, and slightly increased in the secondary polycythaemias, in leukaemia, in most conditions associated with splenomegaly, in some cases of oedema, and during the later months of pregnancy. It is somewhat decreased in most anaemias and in myxoedema, and markedly decreased in shock following acute haemorrhage, and in conditions associated with dehydration. It is normal in essential hypertension and chromic nephritis.

Biopsy examinations frequently give information of great diagnostic value. Sections of enlarged lymph glands, fixed and stained by the usual methods, usually give an immediate, definite diagnosis in cases of Hodgkin's disease, lymphosarcoma, or lymphatic leukaemia, either in the leukaemic or leukopenic stage. Examination of the bone marrow may be equally helpful. A small portion can be removed with a trephine under local anaesthesia, usually from the sternum or tibia. Portions of tissue so obtained should be fixed in formalin, Zenker, or sublimate-alcohol, and cut in thin sections. If stained with Wright's stain, the specific granulations of the leukocytes can be made out. One may also make imprint preparations or thin smears of the marrow on clean cover A better method (Isaacs) is to make a fairly thick suspension of marrow cells in homologous plasma or serum (it must be from the same blood group), by teasing and gently agitating a bit of marrow in a few drops of serum. Films from such a suspension are made and stained in the same way as ordinary blood film. In satisfactory preparations the fine details of cell structure are preserved, and accurate differential counts can be made. A thorough acquaintance with normal marrow is essential if valid conclusions are to be drawn regarding pathological changes.

Such examinations have proved valuable in determining the extent and type of hyperplasia present, both in the anaemias and the leukaemias. It is especially helpful in the diagnosis of leukopenic leukaemia. However, in patients with serious blood diseases such procedures are not without risk, both of infection and bleeding. They should be performed with asepsis as strict as that demanded for a laparotomy.

Young and Osgood (1935) have reported the results of a study of sternal marrow obtained by aspiration by a modification of Arinkin's method (1927). A No. 18 gauge lumbar puncture needle 4 cm. long is used (aseptic precautions, procaine infiltration). With the point at the center of the sternomanubrial junction, the needle is held at an angle of 60° with the chest wall, pointing caudad, and gently forced through the external lamina. Care must be taken not to let the needle jump too deeply into the sternum when the lamina gives way. The base of the needle is then depressed to an angle of 30°, and the needle is gently forced in to a depth of 1 to 1.5 cm. The stylet is removed, a 2 cc. syringe is attached, and 1 or 2 cc. of marrow contents is gently aspirated. If necessary the needle is rotated, and the position of the tip varied until material is obtained. The material, which looks like blood, is expelled into a small tube containing 2 to 3 mg. potassium oxalate per cc. The needle is removed, and the puncture wound sealed with collodion. Films and cell counts are made from this just as from oxalated blood. The cell characteristics are clearly defined.

In a series of 28 normal individuals and 26 miscellaneous patients no untoward results were reported. They found the results especially helpful in the differentiation of simple leukopenia, aplastic anaemia, and aleukaemic leukaemia, and in malaria

reported finding a larger proportion of infected erythrocytes than in the peripheral blood. Dameshek et al. (1937) in a series studied by both methods found the results from biopsy specimens much superior to those from sternal aspirates.

#### COAGULATION FACTORS

Coagulation Time (Lee and White).—(1) In a rack set up 5 small test tubes with an internal diameter of 8 mm. They must be chemically clean, and must be rinsed with physiological salt solution just before the test. In the fourth tube put 3 drops of 1% calcium chloride solution. (2) A sterile 5 cc. (or larger) syringe and needle are rinsed out with sterile physiological salt solution, and the last of the salt solution is expelled with the needle held vertically, so that the needle and the dead space in the tip of the syringe are left filled. (3) Withdraw 5 to 7 cc. of blood from a vein. The validity of the test depends upon entering the vein cleanly at the first attempt. Suction must be gentle to avoid drawing air bubbles into the blood. (4) Remove the needle and gently expel 1 cc. of blood into each tube. In the 5th tube put 2 or 3 cc. if available. (5) After 5 minutes (or less, if a shortened coagulation time is suspected) remove the first tube from the rack, and tilt it gently to determine whether coagulation has occurred. (6) After ½ minute examine the second tube in the same way. (7) After ½ minute more again examine the first tube, and continue alternately at 1/2 minute intervals, until coagulation has progressed so far that the tube can be inverted without spilling the blood. (8) Then examine the 3rd tube, not yet disturbed. If coagulation is not complete, re-examine at 1/2 minute intervals until the tube can be inverted. The total interval from the time the blood first enters the syringe until this tube can be inverted measures the coagulation time.

Normal blood coagulates in from 6 to 12 (or even 15) minutes; more often 12 than 6, if the technique has been perfect. Practically all technical errors hasten coagulation. The coagulation time is longer if tubes of larger diameter are used.

The calcium time is the time required for coagulation to take place in the 4th tube, to which calcium chloride was added. Normally this usually retards coagulation somewhat. If coagulation is delayed in the first 3 tubes and occurs more quickly in the fourth tube, it probably indicates a lack of available calcium, and suggests that therapeutic administration of calcium may be beneficial. Such a lack of calcium is not necessarily associated with a reduction in total serum calcium, as determined by ordinary chemical methods.

Retractility of the Clot.—The fifth tube is put in an incubator and is inspected after 1 hour, and again after 18 to 24 hours. Normally retraction of the clot and expression of serum is appreciable after 1 hour and is marked at the later period. Delayed and incomplete retraction is met with in blood which is deficient in platelets. Occasionally, however, a clot of normal blood fails to separate from the walls of the tube, but if such a clot is loosened with a platinum wire, retraction occurs promptly.

Determination of Coagulation Time with Capillary Blood (Mas y Magro).—In a watch glass coated with paraffin put a large drop of liquid petrolatum. Rinse out a 20 cmm. (Sahli haemometer) pipette with oil. Clean the finger tip with alcohol and ether; puncture deeply enough to get freely flowing drops of blood; wipe off the first drop and fill the pipette from the second drop. Expel this blood gently into the drop of oil and note the time. After two minutes wipe off the tip of the pipette with filter paper and attempt to suck the blood back into the pipette. Repeat every two minutes until sufficient coagulation has occurred to prevent this. The time interval which has

elapsed is the coagulation time. The normal coagulation time by this method is 8 to 12 minutes (less if subsequent drops from the same puncture are taken).

Bleeding Time (Duke).—Make a deep prick in the lobe of the ear or finger. At half-minute intervals remove the drop of blood with a piece of filter paper, taking care not to touch the skin. Note the time that elapses before bleeding ceases. Normally this does not exceed 3 minutes. The bleeding time is prolonged in thrombocytopenia, and in conditions associated with reduction in fibrinogen. It is usually normal in haemophilia.

Capillary Resistance Test (Rumpel-Leeds phenomenon).—Apply a blood pressure cuff about the upper arm and keep inflated for 5 minutes at or slightly above the diastolic pressure. If the test is positive, within a few minutes after the cuff is removed, a crop of purpuric spots will apear in the skin below the cuff. A few small spots are of no significance, but a profuse crop with spots of large size (over 1 cm. in diameter) is almost pathognomonic of thrombocytopenic purpura.

Prothrombin Time (Howell).—(1) Obtain 4 cc. of venous blood, as for testing coagulation time. (2) Put it in a clean centrifuge tube containing 0.5 cc. of a 1% solution of potassium oxalate in 0.85% salt solution. (3) Mix and centrifugalize until the plasma is clear. (4) In each of a series of 6 small, chemically clean test tubes put 5 drops of plasma. (5) Keeping the first tube as a control, add to the succeeding tubes 0.5% calcium chloride solution in quantities increasing 1 drop per tube, from 2 drops in the second tube to 6 drops in the sixth. (6) Mix by shaking gently, note the time, and examine the tubes at 1 minute intervals until coagulation is sufficiently advanced in any one of the tubes so that it can be inverted without spilling the contents. This interval, the "prothrombin time," is normally 10 to 12 minutes. Marked prolongation of the time is met with chiefly in haemophilia. It does not indicate a lack of prothrombin. Coagulation usually occurs first in the second tube, containing the least calcium. If coagulation occurs first in the higher numbered tubes, it suggests a lack of available calcium.

A method for the quantitative estimation of *fibrinogen*, in conjunction with other plasma proteins, is given on page 674.

# Tests for Isoagglutination

### HUMAN BLOOD GROUPS

When the serum of certain individuals is mixed with a suspension of red cells from certain other individuals, agglutination of the cells occurs. By a study of these reactions Landsteiner (1900) was able to divide human beings into 3 distinct groups. The work of his associates, Decastello and Sturli (1902), indicated the existence of a fourth group, which was described as such by Landsteiner (1909). This last publication escaped general notice, and Jansky (1907) and Moss (1910) independently demonstrated the existence of the same four groups, although they gave them different numerical designations. Moss's work was of great practical value because he described a simple method for group determinations which could be used clinically, and because he brought to general notice the practical

importance of the procedure in the selection of suitable donors for transfusion. These discoveries removed the major peril in transfusions and made their extensive use possible for the first time.

The interrelationships between the cells and sera of the four groups can be most readily understood by inspection of the table.

It is evident, first, that red cells are not agglutinated by the serum of another member of the same group. Serum from Group AB (Group I of Moss) has no agglutinating activity, while cells of this group are agglutinated by serum of the other three groups. Cells of Group O (Group 4 of Moss) are inagglutinable, while the serum agglutinates cells of the other 3 groups. Blood from Group A (2) and from Group B (3) cross agglutinate each other's cells, and both also agglutinate the cells of Group AB, while the cells of both are agglutinated by Group O serum.

Table to Show the Relationships of the Cells and Serum of the Different Blood Groups

International Group	Cells AB	Cells A	Cells B	Cells O	Cell Recep- tors	Serum Agglu- tinins	Moss	Jansky Group	Fre- quency, per cent	May give blood to	May receive blood from
Serum Type AB	0	0	0	o	AB	o	I	4	6	AB	All
Serum Type A	+	0	+	o	A	b	2	2	10	A AB	A O
Serum Type B	+	+	0	0	В	a	3	3	10	B AB	B O
Serum Type O	+	+	+	o	0	ab	+	ī	44	All	0

Agglutination is indicated by +, and the absence of agglutination by o.

These observed facts can be explained by means of the following hypothesis. In human serum there may occur two different isoagglutinins, designated arbitrarily a, and b. In human red cells there may occur two corresponding agglutinogens, or receptors, A and B. Any given serum may contain either or both of these agglutinins, or neither of them. Similarly, in a given cell, either or both of the receptors may be present, or neither of them. However, an agglutinin and its corresponding receptor are never both present in the same blood, but one or both of the agglutinins are practically always present if the corresponding receptors are absent. In a mixture of bloods, agglutination will occur whenever an agglutinin and its corresponding receptor are present. The distribution of agglutinins and receptors which is believed to occur is also shown in the table. The correctness of this hypothesis is supported by agglutinin absorption tests.

The characteristics of the cells (possession of receptors) is the factor which determines the group. This is a constitutional trait which is

inherited as a dominant Mendelian character. The group characters are often incompletely developed at birth, especially the agglutinins, but become evident after 3 to 12 months. After these have once developed, the group never changes, although the agglutinating activity of the serum may vary or even disappear. Since the possession of receptors by the red cells is the factor which determines the blood group, it is preferable to designate the groups by symbols which indicate their receptor content, rather than by arbitrarily chosen numerals. The four groups are therefore termed A, B, AB, and O (no receptors).

The table shows the approximate frequency of the groups in the Caucasian races of Europe and North America. In some of the races of Asia and Africa groups B and AB are relatively more frequent, while in American Indians group O is much more frequent. All four groups occur in all the human races which have been studied and, according to Landsteiner, also in the orang.

In about  $\frac{1}{2}$  of the cases a serum which shows agglutinating activity will also haemolyze the red cells, if it is fresh and active. Haemolysis never occurs in the absence of agglutinins, although the process of agglutination may be masked by haemolysis. Inactivation prevents haemolysis, but does not affect agglutination.

The practical importance of blood group determinations depends largely on its use in selecting suitable donors for transfusion. It has been found that blood is suitable for transfusion if the cells of the donor are not affected by the serum of the recipient. On the other hand, if the donor's cells are agglutinated (or haemolyzed) in vitro by the recipient's serum. these cells will be destroyed in the body of the recipient, and will usually cause a violent and sometimes fatal reaction, often with haemoglobinuria and jaundice. While it seems preferable theoretically that the donor and recipient should belong to the same group, in practice it is usually immaterial if the cells of the recipient are agglutinated by the serum of the donor. Unless an unusually large transfusion is given, the activity of the serum of the donor is so modified in the body of the recipient, by dilution or otherwise, that its agglutinative and haemolytic properties are abolished. Such transfusions do not cause reactions more frequently than those in which a donor of the same group is used. With certain limitations to be discussed, a Group O individual can be regarded as a universal donor, since the cells are not agglutinable, while a Group AB individual may be regarded as a universal recipient.

Selection of a Donor.—The donor must be a healthy individual, preferably a young man, free from infectious disease. Syphilis must be excluded with particular care, as the blood in the primary and secondary stages is highly infectious. A Wassermann reaction or a flocculation test should always be done, but a history and complete physical examination are also indispensable. We have seen syphilis conveyed by blood from

a donor with primary syphilis, but a negative Wassermann reaction. The risk is probably small in late tertiary or latent cases. Malaria has been transmitted in a number of instances, even from latent cases, and no one with a history of recent infection should be used as a donor. Other infections the transmission of which has been reported include measles, small pox, influenza, typhoid fever, and tuberculosis.

The blood group of both donor and recipient should be determined. If this indicates that the blood is compatible, check by testing the action of the recipient's serum on the donor's cells. If a universal donor is to be used for the first time for a recipient of a different group, determine the agglutinin titer of the donor's serum for the recipient's cells (see p. 323).

Group Determination. Procedure.—There are required: (1) Serum known to have high agglutinating activity from Group A, Group B, and preferably also Group O. (2) Serum from the patient and each prospective donor. (3) A cell suspension from each, prepared by adding 1 or 2 drops of blood to 5 cc. of a 1.5% sodium citrate solution in physiological salt solution.

Set up the following preparations with cells from each of the individuals to be tested:

- 1. Unknown cells + Group A serum.
- 2. Unknown cells + Group B serum.
- 3. Unknown cells + Group O serum.

The third preparation is not essential, but it serves as a valuable check on the accuracy of the results of the first two preparations.

On a slide mix one drop of serum and one drop of cell suspension. Transfer a small drop of the mixture to the center of a 22 mm. cover slip, invert it over the concavity of a hollow ground slide, and seal with petrolatum. Inspect at once under the low power. The number of red cells should be sufficient to form a single layer, after they have settled to the surface of the drop, with the cells close together but not heaped up. Let it stand in a warm room, and inspect occasionally for an hour, if agglutination does not occur sooner. As a rule agglutination, if it occurs at all, will occur within 5 or 10 minutes and be unmistakable. If it is not definite, agitate the mixture by gently tilting the slide from side to side. The cells will then nearly always settle out, conspicuously and compactly clumped. In preparations in which no agglutination occurs the cells will remain evenly distributed for hours.

With these data, the group can be determined quickly from the following outline. The occurrence of agglutination is indicated by +.

Agglutination in:

Group A serum	Group B serum	Group O serum	Group of cells tested is		
+	+	+	AB		
۰ .	+	+	A		
+	0	+	В		
0	0	0	0		

On the basis of these tests, select a donor belonging to the same group as the patient, or if this is not possible, a group O donor, and set up the following:

4. Recipient's serum + donor's cells.

If this preparation shows no agglutination or haemolysis, the blood is compatible, and the donor may be used.

Vincent's open slide method, recommended by Ottenberg, is preferred by many workers. A full drop of serum and of cell suspension are mixed on a slide, preferably in the concavity of a hollow ground slide. Drying must be prevented, and this can be accomplished by covering the hollow with a cover slip and sealing with petrolatum. The slide is tilted and rotated gently so that the cells are uniformly distributed, and this repeated every two minutes for at least 30 minutes, and preferably for an hour. A mechanical shaker may be used. The preparations are kept at room temperature, and are examined with the naked eye, not with the microscope. True agglutination is easily visible.

The chief sources of technical error are: (r) Weak or deteriorated sera. Their activity must be checked frequently by testing with known agglutinable cells. (2) Haemolysis, which may mask agglutination. The remedy is to inactivate the serum, or use stored serum. (3) Use of too thin or too thick a cell suspension. (4) Failure to agitate the suspension. (5) Confusion of rouleaux with agglutination. The microscopic appearance of the aggregates is usually quite different. Agitation breaks up rouleaux, but intensifies agglutination. Rouleaux formation rarely occurs in stored serum, and it can be prevented by slight dilution of the serum. Except as a source of confusion, rouleaux formation has no significance for transfusion. (6) Dependence upon the agglutinating activity of the serum, instead of using stock sera. (7) Incubation at too low temperatures (see below). (8) The use of serum or cell suspensions which are badly contaminated by bacteria. (Non-specific agglutination may occur.) (9) Gross errors in labeling or in technique.

If stock serum of Groups A and B are not available, the group of a patient may be determined by testing his cells and serum with those from either a known Group A, or a Group B individual, thus:

Serum A and X cells	(From is		Serum B and X cells	Serum X and B cells		
+ 0 +	o +	AB AB	+ + 0	0 +		
0	+	0	0	+		

The disadvantage is that errors may occur if the agglutinating activity of either serum happens to be feeble.

If neither stock sera nor individuals of known group are available, in emergencies one must depend on preliminary "cross-matching" tests for the selection of a donor.

Because of the difference in the frequency of occurrence of the different Groups, it is possible, by examining a considerable number of individuals, to determine the Group of each, even if no known type is available. Select at random 20 individuals (preferably Caucasians from Europe or America), test the serum and cells of each with the cells and expect to find about 1 in Group AB, 8 in Group A, 2 in Group B, and 9 in Group O. Those individuals whose cells are not agglutinated in any of the preparations may

be recognized at once as belonging to Group O. The others (A, B, or AB) may be assembled in 2 or 3 groups by putting into individual groups those whose cells-serum mixtures show no cross agglutination with each other. (In such a small number of individuals, the rare Group AB, and even Group B, may happen not to be represented.) If one of these groups markedly outnumbers the others, it may be accepted as Group A.

If there is any doubt, a further check is available. Agglutinogen A is serologically related to the Forssmann antigen, and cells containing A are agglutinated by any serum containing the heterophile agglutinin, as for example, the rabbit anti-sheep serum commonly used in the Wassermann reaction. The species-specific heteroagglutinin for human cells (not specific for A) must first be removed by absorption with Group O cells (which do not contain A). By titrating the serum (after absorption) with cell suspensions from different individuals, significant agglutination of cells containing A will occur (Groups A and AB).

When a transfusion of 500 cc. is given to an adult of average size, the blood of the donor is diluted about 1 to 10 in that of the recipient. Not infrequently the blood of a Group O donor shows an agglutinin titer substantially higher than 1 to 10. On the assumptions that reactions to incompatible blood are due to agglutination of red cells in the body, and that the usual absence of such reactions when Group O individuals are used as universal donors is due purely to dilution, many writers have emphasized the theoretical danger of using a universal donor whose serum has a high agglutinin titer, and have called them dangerous universal donors. There is, as a matter of fact. no positive evidence that either of these assumptions is true. Furthermore, clinical experience indicates that reactions which can be ascribed to this are excessively rare, in spite of the frequency with which universal donors have been used without regard to the titer of their serum. With massive transfusions, however, the risk is a real one, and even with transfusions of 500 cc. no reasonable precaution should be neglected.

To test the titer dilute one part of the donor's serum with 4 parts of salt solution. Mix one drop of diluted serum with one drop of the recipient's cell suspension. Observe for 10 minutes, as above. If appreciable agglutination occurs, secure another donor.

In determining the blood group one should remember that the agglutinins in a serum may be weak or absent. The agglutinability of the cells however, remains constant. For this reason, except perhaps in emergencies, one should not depend entirely on testing the action of the recipient's serum on the donor's cells. We have seen a number of violent reactions occur after injections of Group A blood into a Group O patient whose serum had shown no agglutinating activity, and who was supposed to "match," on the basis of direct tests alone. Whenever there is the slightest doubt as to the group, use a known universal donor.

The U. S. Veterans' Administration (Bull. No. 26) in the selection of donors carries out both group determinations and direct matchings. A drop of each stock serum (O, A and B) is put on a slide and a loop of whole blood from a finger puncture is mixed with each. The slide is tilted repeatedly and agglutination observed macroscopically. A drop of serum from donor and one from recipient are put on a slide, and mixed with a drop of cell suspension (one drop of blood in 0.5 cc. of 1.5% sodium citrate solution) from recipient and donor, respectively. The preparations are similarly observed.

If a donor from the same group as the patient is not available, a universal donor (Group O) is used. The agglutinin titer of his serum is first tested. Defibrinated whole blood from the recipient is drawn up to the 0.5 mark in the stem of a white cell pipette, and then salt solution until the 1.0 mark is reached. This is expelled onto a slide. Donor's serum diluted with an equal volume of salt solution is drawn up to the 0.2 mark and mixed with the drop of cell suspension on the slide. A cover slip is applied and the preparation observed under the microscope for 20 minutes. If no agglutination occurs, the donor is regarded as acceptable.

Preparation of Stock Serum.—Secure sterile serum from individuals known to belong to Group A, B, and (preferably also) O, whose serum has been shown to have active agglutinating power. (1) Seal one drop of sterile serum (enough for a single test) in each of a number of sterile capillary glass tubes. Avoid overheating the serum in sealing.

Or, (2) add to each 9 cc. of clear serum, 1 cc. of 15% sodium citrate solution in physiological salt solution. To each 9 cc. of this citrated serum add 1 cc. of 5% solution of phenol in glycerin. Such serum keeps well for several months. Label with care, and handle each serum separately to avoid accidental confusion of the groups.

Sub-groups, in the sense of clear cut subdivisions of the major blood groups, have been demonstrated only in the case of Groups A and AB. If properly selected Group B or Group O sera are titrated as to their agglutinative activity with the cells from many Group A individuals, the latter are found to fall quite sharply into two subgroups. One possesses cells which are agglutinable in relatively high dilutions of serum, while cells of the other are agglutinated only in relatively high concentrations of serum. The titer for cells of the first group ("Typus A gross") may be 4 to 8 times that for those of the second subgroup ("Typus A klein"). The work of Landsteiner et al. (1926, 1929) indicates that the greater agglutinability is associated with an additional receptorelement in the cells, to which he gives the designation A' (the C of Guthrie and Huck). The readily agglutinable subgroup AA' is about three times as frequent as the other (A, or AA<sup>2</sup> as it is designated by Landsteiner). Because A' occurs only in conjunction with A, and for other reasons, he regards the subdivisions as of only minor significance. There is no proof that individuals in the two different subgroups are not compatible for purposes of transfusion, and much presumptive evidence that they are. The point of practical importance is that rarely a stock serum may be obtained which will agglutinate Group AA' cells readily, but not Group A cells. With such a serum a group A individual may be mistaken for a Group O, and used as a donor for a Group O recipient, with serious results. Group AB may also be subdivided into AB and AA'B.

Minor Agglutinins; Cold Agglutinins.—If one prepares many mixtures of sera with cells from other members of the same blood group, or with Group O cells, and keeps them at ice box temperature, in a limited number of the cases agglutination will occur. These reactions are dependent on the interaction of a number of different agglutinin-receptor pairs. Their incidence appears to be entirely independent of that of the four major groups. Thus far no clear-cut subgroups have been outlined on the basis of such reactions.

As a rule this agglutination is feeble, it occurs only in high concentrations of serum, and it disappears if the preparations are warmed. No corresponding haemolysins have been found. In some cases, however, the agglutination is marked, it may be present in diluted serum, and rarely it persists in lessened degree at higher temperatures. It may then cause errors in group determinations. These agglutinations were responsible for

the multiplicity of groups described by Guthrie and Huck. Several observers, without adequate proof, have regarded these agglutinins as a cause of some of the reactions following transfusions. It is very doubtful if this is true except in the rarest instances. Landsteiner observed 5 transfusions without reaction, in which the recipients' sera showed a high content of "cold agglutinins" active on the donors' cells. However, in the Johns Hopkins Hospital a severe reaction has recently been observed in a patient whose serum showed marked agglutinating activity of this type.

Autoagglutination.—If mixtures of serum and cells from the same individual are kept at ice box temperature, in some cases agglutination occurs. The clumps break up as soon as the mixtures are warmed. Such sera will similarly clump any red cells (human or animal) added to it; i.e., it is a "panagglutination." Slight degrees of such agglutination are said to be common, but a marked degree of agglutination is rare. It has been reported in acquired haemolytic jaundice, in cirrhosis and other diseases of the liver, in chronic sepsis, leukaemia, pernicious anaemia, etc. One of us (M.C.C.) has observed it in high titer in a woman with myocardial insufficiency and in a healthy daughter, apparently as a familial trait. It has little if any practical significance.

The autohaemolysin of paroxysmal haemoglobinuria is described in a following chapter.

### Medico-legal Applications

Disputed Parentage.—Since the receptors A and B are inherited as independent dominant Mendelian characters, it follows that a receptor can not appear in a child unless it is present in one of the parents. Therefore, if the group of the child and of one parent is known, it is possible in some cases to determine that a given individual can not be the other parent. This subject has been further elaborated by Bernstein, (1924) who advanced the theory that the inheritance of the blood groups is determined by the distribution of 3 allelomorphic genes; A, B, (dominants), and R (or O), a recessive (negative). One gene is present in each member of a pair of chromosomes, so that each somatic cell possesses two of these genes, and each sex cell, one gene. The genetic structure of the four groups would, therefore, be: Group O. OO: Group A. AA or AO: Group B, BB or BO; Group AB, AB. Since a child draws one gene from each parent. it follows that a Group O parent can never have a Group AB child, and a Group AB parent can never have a Group O child, regardless of the group of the other parent. With any other combination of groups in the parents, a child may have O genes only, or either A or B or both, provided these are present in either parent. Recent careful studies of large numbers of families have confirmed these assumptions, and their validity has been Definite information is obtained in from 20% to 30% of the disputed situations.

Landsteiner et al. (1928) have demonstrated two other definite agglutinogens in human blood, which he has designated as M and N. There are no corresponding agglutinins in human serum, and their demonstration is a highly complicated procedure requiring the blood of specially immunized animals. Their distribution is entirely independent of that of the four major groups. One or both of them are present in every human blood. Like agglutinogens A and B, M and N are inherited as independent dominants, and human beings can be divided on this basis into 3 classes or "groups," having a theoretical genetic structure of MN (50%), MM (30%) and NN (20%). Their presence can also be utilized to decide questions of disputed parentage. This gives definite information in about an additional 30% of the cases.

Determination of the Group in Blood Stains.—If the blood dried before decomposition occurred, and if the proteins have not been denatured by heat or otherwise, it is usually possible to determine the group of the individual from whom the spot was derived. It is useless to attempt direct agglutination of cells recovered from such material, and only occasionally is it possible to demonstrate agglutinins directly in extracts of such spots. The only practicable method is based upon the fact that the isoagglutinins are specifically "absorbed" and removed from a serum when brought into contact with red cells containing the corresponding receptor, or with the products of haemolysis of such cells, or with seminal fluid or tissue juices of individuals belonging to the same group.

Method.—(1) Secure A serum and B serum with a high agglutinin titer (preferably 1 to 160), and dilute each 5 times with salt solution.

(2) Into each of two centrifuge tubes put 20 to 40 mg. of dried blood, or cut into fine bits a piece of stained cloth (if heavily blood-stained 1 sq. cm. suffices), and put half into each tube. (3) Add 0.5 cc. of salt solution to each, and let stand one hour. (4) To tube 1 add 0.5 cc. of diluted A serum, and to tube 2, 0.5 cc. of diluted B serum, and let stand 3 hours. (5) Centrifugalize, remove the clear fluid which contains serum in 1 to 10 dilution, and set up a series of dilutions of each, in 0.5 cc. volume, from 1 to 10 to 1 to 160. (6) To each tube in the first series (containing A serum) add 2 drops of a 5% suspension of washed Group B cells; add to each tube in the second series, a similar quantity of A cells. (7) Read after 2 hours.

If agglutination occurs as indicated, the group of the blood in the spot is:

A cells	B cells	Group is
+	0	В
0	+	A
+	+	0
•	0	AB
•	0	AB

To ensure the certainty in the results needed for medico-legal purposes, elaborate controls are necessary, including parallel absorption tests with blood spots of known groups. By this method the group has been determined in blood spots 15 to 20 years old.

Substitutes for Blood.—To replace blood lost by acute haemorrhage or to fill the vessels adequately in cases of surgical shock the best fluid is blood; but if a donor is not available or if from the standpoint of military efficiency it seems unwise to solicit donors, some other fluids must be utilized. Such fluids may be of great value since the cause of death in such conditions is not lack of erythrocytes but insufficient fluid to fill the blood vessels adequately to maintain blood pressure.

Considerable latitude is possible in the choice of fluids which may be injected intravenously with safety. Among those most readily available are physiological salt solution, Locke's solution, Ringer's solution, glucose solution, or various modifications of these. Such solutions, however, are unsatisfactory and relatively ineffective because both the salt and the fluid are eliminated with great rapidity.

To remedy this defect Bayliss (1916) suggested the use of gum acacia in salt solution. The acacia is excreted slowly and the fluid is retained. In spite of theoretical objections,

careful preliminary experiments on animals and man seemed to indicate the harmlessness and relative effectiveness of the procedure. However, under actual working conditions, probably because of faulty technique, severe and even fatal reactions were reported after its use. More recent work, notably that of Huffman (1020) at the Mayo Clinic, has confirmed that of Bayliss and shows that if properly prepared the acacia solution is not toxic and is valuable in the treatment of surgical shock.

He prepared the solution as follows: In a four-liter beaker put 480 Gm. of acacia (lumps of No. 1 extra quality) and 72 Gm. of sodium chloride (C.P.). Add 500 cc. of triply-distilled water and heat over a water or steam bath, stirring with a glass rod until a considerable amount of the gum is dissolved. Decant the thick solution, add 500 cc. more water to the residue and again extract, repeating until all the gum is dissolved. Dilute the combined extracts to 8 liters in a large flask and sterilize in the autoclave. Clear the turbid solution by filtration through soft paper in a Buchner funnel. Resterilize twice more (a precipitate forms after each resterilization). Then clear by sedimentation, and siphon off the clear supernatant fluid into sterile bottles, using aseptic precautions. If stoppered tightly it keeps for several months. Test sterility, and check toxicity by injection into animals (dogs or rabbits).

When administered to man the rate of injection should not exceed 20 cc. per minute. It should be kept at body temperature. The injection is stopped after the blood pressure has risen adequately or after a maximum of 800 cc. has been given. If necessary two or even three injections can be given within 24 hours.

Preservation of Blood for Transfusion.—Blood which has been drawn into sodium citrate solution (70 cc. of a 2.5% solution per 500 cc. of blood; may be preserved for three to four weeks by storing it in the ice box at 4°C. The group should be determined, a Wassermann test carried out, and cultures made to prove sterility. Before use, a cross-matching with the blood of the prospective recipient should be carried out. The experience with the use of stored blood at the Cook County Hospital, Chicago (Fantus, 1937), shows that such blood may be employed without danger of reactions until haemolysis of the red cells begins. When the first trace of haemolysis appears, the colorless plasma may be siphoned off and used as an effective substitute for whole blood in such conditions as postoperative surgical shock or extensive burns, in which there is no anaemia and the need is for additional fluid and not for red cells.

Cadaver Blood.—Yudin and his associates at Moscow (1932, 1937) have reported the successful use of blood obtained from cadavers within six to eight hours after death. Blood (two to three liters) was drawn aseptically from the jugular vein. It might be mixed with citrate solution in the usual way. If, however, the blood was obtained from individuals who had died suddenly, either from accidents or from such conditions as coronary thrombosis, and was allowed to coagulate, the clot would speedily undergo fibrinolysis, and the re-liquefied blood would not again clot. A Wassermann reaction cultures and a complete necropsy were carried out to exclude conditions which might contraindicate its use. (In warm climates, thick blood films and films from the spleen and bone marrow would also be required, to exclude malaria.) They found that such blood could be preserved for three to four weeks by storing it at r° to 2°C. It was as effective and caused fewer febrile reactions (5% as compared with 20%) than fresh citrated blood from living donors.

# CHAPTER XIV

# NORMAL AND PATHOLOGICAL BLOOD CELLS

### RED CELLS

The normal values for red cells of young adults are given in the following table (slightly altered from Wintrobe).

		Male	Female		
	Average	Range	Average	Range	
Red cell count in millions		4.6 to 6.2 14 to 18 40 to 54	4.8 14.2 42	4.2 to 5.4 12 to 16 37 to 47	

	Average	Range
Mean corpuscular volume in cubic micra  Mean corpuscular haemoglobin in micro-micro-grams  Mean corpuscular haemoglobin concentration in %	30	82 to 92 28 to 32 32 to 36

The red cell count in young infants is usually about 5.5 to 6.0 million. It falls rapidly during the first few weeks to 4.0 to 5.0 million, and during childhood remains somewhat lower than the adult figures. The red cell count may fluctuate during the day within a range of at least 10%, with changes in plasma volume, or with the storage of red cells in, or their release from the spleen.

The blood of the normal young male adult in this country has an average O-combining capacity of 20.9 volumes per cent (Van Slyke), corresponding to 15.6 Gm. per 100 cc. In Great Britain the normal figure is usually given as 14.5 Gm. The variations with age and the daily fluctuations parallel those noted for the red cell count.

For convenience in calculating the indices it is customary to regard 5.0 million red cells per cmm. as the normal figure, and the quantity of haemoglobin in normal blood having exactly this red cell count as the normal figure for haemoglobin (Osgood's haemoglobin coefficient). On this basis 14.5 Gm. of Hb. corresponds to 100%. Similarly, the average volume of packed red cells in normal blood having a red cell count of 5.0 million per cmm. is 42% (the volume coefficient of Osgood).

The mean corpuscular volume (Wintrobe) in cubic micra is calculated by dividing the volume of packed red cells, expressed in cc. per liter, by the red cell count in millions. The mean corpuscular haemoglobin in micro-micro-grams  $(\gamma \gamma)$  is calculated by dividing

the Hb. content of the blood, expressed in grams per liter, by the red cell count in millions. A micro-micro-gram is one millionth of one millionth of a gram. The mean corpuscular haemoglobin concentration in per cent is calculated by dividing the Hb. content of the blood, expressed in Gm. per liter, by the volume of packed red cells, in cc. per liter, and multiplying by 100. These are absolute values, and not dependent numerically on any arbitrary standard of normal.

These relationships are more commonly expressed as indices.

The color index (color-count ratio) expresses the relative amount of Hb. in each red cell, as compared with that of normal blood. It is obtained by dividing the Hb. content of the blood by the red cell count, both expressed in percentages of the normal figures. The percentage of the normal red cell count is obtained simply by multiplying the first two figures of the count by 2.

The *volume index* (volume-count ratio) expresses the relative volume of the individual red cell, as compared with that of normal blood. It is calculated by dividing the red cell volume by the red cell count, both expressed in percentages of the normal figures.

The saturation index (color-volume ratio) expresses the relative concentration of Hb. in the red cells, as compared with normal blood. It is calculated by dividing the Hb. by the red cell volume, both expressed in percentages of normal. It may be diminished, but it is never increased.

The average normal value for each index is, by definition, 1.0, and the normal range is at least from 0.9 to 1.1. The great importance of these relationships in the classification and diagnosis of anaemias is discussed in the following chapter.

Example.—The normal values commonly used for these calculations are: Red cell count 5.0 million. Hb. 14.5 Gm. per 100 cc. Cell volume 42%. Assume that a given blood shows:

```
Red cell count 2.5 million = 50% of normal.

Hb. is 8.7 Gm., = 60% of normal.

Cell volume is 30%, = 71% of normal.

Then the color index = Hb./count = ^69_{50} = 1.2

The volume index = Volume/count = ^71_{50} = 1.4

The saturation index = Hb./volume = ^69_{11} = 0.86

The mean corpuscular volume = ^300/2.5 = 120 cubic micra.

The mean corpuscular Hb. = ^87/2.5 = 35 micromicrograms.

The mean corpuscular Hb. concentration = ^87_{500} = 29%.
```

Morphology of the Red Cells.—In normal blood the red cells are all circular in outline, and nearly uniform in size, with a mean diameter of about 7.5 micra (7.2, Price-Jones). Two thirds of the cells are from 7 to 8 micra in diameter. An occasional cell may be as small as  $5.5\mu$ , or as large as  $9.5\mu$ . A greater diversity in size is called anisocytosis. Cells which are less than  $5\mu$  in diameter are termed microcytes; those

over 10 $\mu$ , macrocytes. Cells which are abnormal in shape, like a sausage, battledore, or dumbbell, poikilocytes. Most of the microcytes and poikilocytes are probably portions of fragmented cells.

With or without marked diversity in size, the average size of the red cells may be abnormally small (microcytosis) or abnormally large (macrocytosis). The determination of the mean corpuscular volume or volume index gives a better indication of the average size of the cells than merely a measurement of their diameter, but in most conditions there is a rough parallelism between them.

The depth of color of the cells, either in fresh or stained films, is nearly as constant as their size. Abnormal variations in color (anisochromia) are usually due to variations in thickness of the cells. Unusual pallor of the cells ("achromia") may be due in part to diminished concentration of Hb. in the cell. Such cells may have colorless centers with only a narrow yellowish rim of Hb.-containing stroma ("pessary forms").

Immature red blood cells are found in any form of anaemia in which there is active regeneration of new cells. They may be regarded as hurried into the circulation before development is completed, to satisfy an urgent need for cells. The following types of red cells are regarded as immature.

Polychromatophilia, polychromasia, diffuse basophilia.—In films stained with any type of Romanowsky stain a limited number of the red cells may show a more or less marked degree of diffuse bluish staining. The term punctate basophilia or stippling (Grawitz granules) is applied to red cells showing discrete, bluish-black granules with such stains. They are especially numerous in lead poisoning. These granules are regarded as evidence of degenerative changes in the cytoplasm of immature cells.

Reticulocytes in increased number constitute the most constant and dependable sign of active regeneration in anaemia of any type. In vitally stained films they contain bluish granules which, in the more immature cells, are numerous and connected with one another by a meshwork of fine bluish strands. The more mature reticulocytes contain a few similar granules, or even only a single granule, without any visible strands, and are visible only in the best preparations. The granules are thought by some to be mitochondria. They are not identical with the granules in ordinary stippled cells. Reticulocytes are more numerous than stippled cells, and may be numerous in blood which contains no stippled cells. An abrupt rise to a peak of 5% to 40% or more, a reticulocyte crisis, occurs within a few days after the institution of effective treatment in severe cases of pernicious anaemia or hypochromic anaemia. Reticulocytes are especially numerous in familial haemolytic jaundice (frequently 20%, rarely even 50%).

### Nucleated Red Cells

Normoblasts are approximately the size of a normal red cell or slightly larger. The more nearly mature forms contain a nucleus which has a diameter from ½ to ½ that of the cell, which is round, and which stains a dense, homogeneous, purplish-black color, without showing any structure (pycnotic). The nucleus may be lobulated or fragmented. The cytoplasm may be normal or basophilic in varying degree. The "immature normoblasts" tend to be somewhat larger and have a more basophilic cytoplasm. The nucleus shows definite structure and usually contains coarse, deeply stained, wedgeshaped masses of chromatin separated by radiating, pale lines, which give it a "wheelspoke" appearance.

The more primitive forms ("macroblasts"), seen only in severe anaemias, have nuclei with a finer chromatin structure, but they usually show the wheel-spoke arrangement

of the chromatin more or less definitely. The cytoplasm is usually strongly basophilic and somewhat more abundant.

Megaloblasts are the most primitive red cells which appear in the circulation. They are large cells, 10 to 20μ or more in diameter. The nucleus is about as large as a normal red cell, it is approximately central, and there is no pale perinuclear zone. It shows a distinct nuclear membrane and contains interlacing strands of fine chromatin masses. The most primitive may contain nucleoli. Rarely mitotic figures may be found. The cytoplasm may be polychromatophilic, or it may stain intensely blue, without any suggestion of haemoglobin. Usually, however, it has a greenish tinge. It looks waxy, homogeneous, and uniformly thick out to the extreme margin of the cell. This point helps to distinguish them from primitive leukocytes, which usually have thin and somewhat irregular margins. They rarely occur in adults in typical form except in severe untreated cases of pernicious anaemia. However, the view of Ehrlich. Naegeli, etc., that they are pathognomonic of pernicious anaemia and are fundamentally different from the erythroblasts seen in other diseases is no longer generally accepted.



Fig. 70.—Nuclear particles or Howell-Jolly bodies in red corpuscles. From a case of pernicious anaemia. Wright's stain ( $\times$ 1000). (*J. C. Todd*, "Clinical Diagnosis.")

Nuclear particles differ from the granules previously described in that they take a purplish-red color with Wright's stain. They may occur as fine granules, usually clustered about the periphery of the cell, chromatin dust. When they are in the form of coarse granules they are called Howell-Jolly bodies. They may also occur as filaments which are variously looped or curled, (Cabot's rings). These structures may be found in any condition in which nucleated red cells are present and have about the same significance. They are usually sparse, but may be numerous for many months after splenectomy, even in the absence of anaemia.

# THE WHITE BLOOD CELLS

The total leukocyte count in normal blood ranges from 5,000 to 10,000 per cmm. Variations as great as this may be observed in the blood of the same normal individual from day to day, or at different hours of the same day.

The leukocytes are made up of three distinct groups of cells which differ from one another fundamentally in origin, in structure, and in function. These are the granular (myeloid) leukocytes, or granulocytes, the lymphocytes, and the monocytes.

The appearance of the cells in fresh, supravitally stained films has been described in the previous chapter. The following descriptions apply to films stained by Wright's or Giemsa's method.

The granulocytes contain definite granules which are visible in fresh, as well as in stained preparations. In normal blood there are three varieties, distinguished by the staining reaction of the granules: neutrophiles, eosinophiles (acidophiles), and basophiles.

The polymorphonuclear neutrophiles show a faintly pinkish-tinged cytoplasm filled with nearly uniform, fine granules which take a pink, lilac, or (with alkaline stains) a bluish-violet color. The nucleus is usually divided irregularly into from two to five lobes which are connected by thin filaments of chromatin. There is a distinct nuclear membrane. The chromatin is arranged in coarse strands which stain a deep purplish-blue color. There are no nucleoli. In about 4% of these cells the nucleus is band shaped, or segmentation is incomplete, but the chromatin is in coarse strands and is densely stained. These are the "non-filament" forms, or the Stabzellen of Schilling.

The polymorphonuclear eosinophiles are distinguished by the coarse, uniform, highly refractile granules which stain a bright vermilion-red color. The nucleus is usually bilobed, larger, and finer in structure, and stains less intensely than that of the neutrophiles.

The polymorphonuclear basophiles (mast cells) contain purplish or bluish-black granules which are usually intermediate in size between those of the preceding types of cells, and are less refractile than the eosinophile granules. They tend to vary in size and depth of staining, and are often sparse. The nucleus stains more faintly, and the lobulation is often indistinct.

Immature granulocytes, the normal ancestors of the preceding types of cells, may appear in the blood in certain pathological conditions. Normally some mechanism effectively prevents the passage of cells into the blood before their development is complete. It is the phenomenon of their entrance into the blood stream, and not the character of the cells themselves, which is pathological. In general the more numerous and the less mature these cells are, the more serious is the disturbance of the haematopoietic tissue.

Myclocytes are the immediate precursors of the granulocytes. The more nearly mature forms resemble the mature granulocytes in their cytoplasm and specific granulations. There are neutrophilic, eosinophilic, and basophilic myelocytes. They differ from the mature cells in having round or oval nuclei, with little or no indentation. The nucleus takes a paler stain, and shows a finer chromatin network. There are no nucleoli. The myelocytes are non-motile and not phagocytic, an important point in distinguishing them from monocytes.

Metamyelocytes are cells which are intermediate in development between myelocytes and mature neutrophiles. The nucleus has a moderately fine chromatin network, and

is deeply indented, or has the shape of a broad band (the Jugendformen of Schilling). An occasional cell of this type may rarely be found in normal blood.

The less mature myelocytes differ in having a bluish rather than a pinkish cytoplasm, although a small area next to the nucleus may be pinkish. The granules are not differentiated, and take a bluish stain, or in the same cell some may be lilac or red, and some blue. They are relatively scanty. The nucleus has a fine chromatin network, and may contain nucleoli. The cells are often much larger than a mature granulocyte. The most primitive myelocytes, the promyelocytes (A-myelocytes of Sabin), have a deeply basophilic cytoplasm, and contain only a few (or even a single) bluish granules, or small purplish-red rods (Auer bodies) which take the peroxidase stain. The nucleus is like that of the myeloblast.

The mycloblust is the earliest recognizable precursor of the myclocytes. It is rarely seen in the blood except in the acute stages of leukaemia. The cell is usually large, and has a large, somewhat eccentric, round or oval nucleus. This has a fine chromatin network which takes a reddish purple color and gives the nucleus a velvety or finely stippled appearance. There are usually several (2 to 5) nucleoli, variable in size and shape, and pale sky-blue in color. They show no definite limiting membrane, and look like irregular lacunae in the chromatin network. There is no distinct nuclear membrane. Exceptionally the nucleus may be indented, convoluted, or irregularly lobulated. The cytoplasm is deeply basophilic, darker than the nucleus, and (by definition) contains no granules. There is no perinuclear clear area.

These cells are difficult to differentiate from lymphoblasts and other primitive leukocytes, and in some cases it is impossible to do so with the method's of study now available. Such undifferentiated cells are sometimes termed stem cells, and are regarded by some as common ancestors of all the types of leucocytes.

In some cases small myeloblasts with dense, heavily staining nuclei occur, practically indistinguishable from lymphocytes. Presumptive evidence that questionable cells are myeloblasts may be obtained by demonstrating the occurrence with them of myelocytes (containing peroxidase-staining granules).

The Lymphocytes in the blood of a normal adult are nearly all (mature) small cells, about the size of a red cell. The nucleus is relatively large, round or oval, eccentric, and often notched, or rarely, deeply lobed. It contains irregular, coarse, dense masses of chromatin which take a deep violet-blue stain. These masses are not sharply demarcated from the pale-staining parachromatin of the nucleus. There may be one or two nucleoli, visible only in crushed or flattened cells. The cytoplasm is sky blue or deep blue, but paler than the nucleus, and it usually shows a narrow crescentic pale zone next to the nucleus. No granules are present in fresh preparations, but in stained films about one third of the cells show a few reddish-violet "azure" granules. Lymphocytes never show granules with the peroxidase stain.

Large lymphocytes differ chiefly in having more abundant cytoplasm. The nucleus is somewhat larger and usually takes a paler stain. The chromatin masses are often finer, and such cells are less mature than the normal small lymphocyte. These cells occur in small numbers in adults, and are common in children. There is little to be gained by separating large from small lymphocytes in the differential count.

Lymphoblasts, primitive lymphocytes, are large cells with abundant cytoplasm which usually is strongly basophilic. It often shows a perinuclear pale zone, and may contain azure granules. The nucleus stains less intensely, and shows fine chromatin masses, although these are usually coarser than in the myeloblast. There are usually

No. 1. Polymorphonuclear neutrophile.

No. 2. Polymorphonuclear basophile. The lobes of the nucleus are indistinct and are partly overlaid with granules. The latter vary in their size and depth of staining.

No. 3. Polymorphonuclear eosinophile. The granules are large and uniform in size.

No. 4. Clump of normal platelets.

No. 5. 'Small lymphocyte, with a "wheel-spoke" arrangement of the chromatin in the nucleus. There is a definite clear zone in the cytoplasm around the nucleus.

No. 6. Small lymphocyte, containing azure granules. The chromatin is in large masses.

No. 7. Large lymphocyte, containing azure granules. The chromatin is in coarse masses. There is an indistinct nucleolus. There is a pale zone in the cytoplasm around the nucleus.

Nos. 8 and 9. Monocytes. The cytoplasm is greyish blue in color. There is a fine lilac granulation which is abundant but does not stuff the cell as does that of the granulocytes. The nuclear chromatin is arranged in a fine network as contrasted with the coarse skeins of the granulocytes and the dense masses of the lymphocytes. There are no nucleoli. No. 9 is the transitional of Ehrlich.

No. 10. Plasma cell. The cytoplasm is abundant, basophilic, with two vacuoles and a small pale perinuclear zone. The chromatin masses are coarse and show the "wheel-spoke" arrangement.

No. 11. Giant platelet, from a case of pernicious anaemia.

No. 12. Neutrophilic metamyelocyte, with band-shaped nucleus.

No. 13. Neutrophilic myelocyte, with oval nucleus and practically mature granulations.

No. 14. Primitive type of myelocyte, with sparse, bluish, undifferentiated granules. The cytoplasm is diffusely basophilic except for a narrow acidophilic zone at the left of the nucleus. The nuclear structure is fine, and there are two nucleoil.

No. 15. Myeloblast. There is no distinct nuclear membrane, the background of the nucleus is finely stippled, and there are five nucleoli. The cytoplasm is deeply basophilic, is free from granules and shows no pale perinuclear zone.

No. 16. Premyelocyte. Essentially identical with the myeloblast except for a few reddish rod-shaped structures (Auer bodies) and bluish granules.

No. 17. Lymphoblast. The cytoplasm is deeply basophilic, with a perinuclear pale zone. There is a definite condensation of chromatin in the nuclear membrane and around the two nucleoli. The chromatin is appreciably coarser than in the myeloblast.

No. 18. Giant neutrophilic leukocyte, with hypersegmented nucleus from a case of pernicious anaemia.

No. 19. "Toxic" neutrophile, from a patient with a severe infection, showing degenerative changes. The nucleus is swollen, with loss of the finer markings. The cytoplasm is vacuolated, and the granules are sparse, and variable and abnormal in size and tint.

No. 20. Rieder cell. A primitive lymphocyte with basophilic cytoplasm and a convoluted or lobulated nucleus.

No. 21. Megaloblast. An early type. The structure of the nuclear chromatin is fine and there are two nucleoli. The cytoplasm has an opaque greenish blue color, but shows a slight tendency to become acidophilic near the lower pole of the nucleus. The margins of the cell look thick.

No. 22. Megaloblast. A late type, with acidophilic cytoplasm and a nucleus which is becoming pycnotic and is undergoing a curious type of fragmentation.

No. 23. Megaloblast. An early type, with basophilic greenish cytoplasm. The nucleus has a relatively fine chromatin network, but somewhat coarser than in No. 21.

No. 24. Immature normoblast, with faintly basophilic cytoplasm and characteristic wheelspoke arrangement of the nuclear chromatin.

No. 25. Megaloblast. The nucleus is undergoing mitotic division. The cytoplasm shows fine basophilic stippling. These four megaloblasts were drawn from the blood of an advanced case of pernicious anaemia who had had no liver therapy.

No. 26. Reticulocyte, vitally stained with cresyl blue.

No. 27. Normoblast, with a pycnotic nucleus and fine basophilic stippling.

No. 28. Red cell, with a large nuclear fragment and fine "chromatin dust."

No. 29. Red cell, showing basophilic stippling and a Howell-Jolly body.

No. 30. Red cell, showing a Cabot ring. The cytoplasm is diffusely basophilic.

No. 31. Megakaryocyte, from a case of pernicious anaemia. The cytoplasm is sky-blue, with small clumps of bright red granules, identical in appearance with those of the platelets. The nucleus may be round or oval rather than elongated, as in this cell. Wilson's stain.  $\times$  1200.

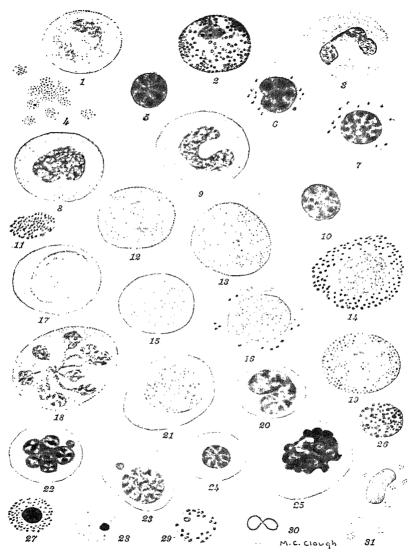


Fig. 71.—Types of normal and pathological blood cells. (For descriptive legend see page 334.)



2 or 3 nucleoli with a dense nucleolar membrane. The most primitive forms are practically indistinguishable from myeloblasts. Lymphoblasts occur chiefly in acute cases of lymphatic leukaemia, but a few may be found in acute infections, or other conditions in which the production of lymphocytes is stimulated. The term *Rieder cell* has been applied to lymphoblasts with lobulated nuclei.

. Wiseman has described progressive changes in the lymphocytes which he regards as evidence of increasing maturity. He stresses the disappearance of nucleoli, decrease in number and disappearance of mitochondria, decrease in the degree of basophilia of the cytoplasm, and finally pycnosis of the nucleus and rupture of the cell membrane. Others question the reliability of basophilia as a criterion of immaturity in lymphocytes, since this does not always parallel those features of the nuclear structure (particularly fineness of the chromatin masses) which they regard as most significant.

Plasma cells are large cells with abundant, deeply basophilic cytoplasm which often contains azure granules, and may contain vacuoles. There is usually a pale perinuclear area. The nucleus is eccentric and contains coarse, dense masses of chromatin which tend to show a radiating, wheel-spoke arrangement. Immature plasma cells with nuclei of fine texture may occur in leukaemia. Plasma cells have been regarded generally as lymphoid in origin, but they are not immature lymphocytes. Some believe them to be a distinct and independent type of cell, related to Türck's "irritation" cells. They are rare in normal blood, but a few may be present in any condition in which there is a lymphocytosis, especially in German measles, infectious mononucleosis, and in a few cases of multiple myelomata.

Türck's irritation cells are large cells with deeply basophilic cytoplasm, which is often vacuolated, but contains no granules. They are regarded by Naegeli as plasma cells, or pathological lymphoblasts, because they have a lymphocytic type of nucleus. This is usually finer in texture than that of the plasma cells. They may occur in any condition in which leukocyte formation is markedly stimulated.

Monocytes are large cells with abundant greyish-blue cytoplasm which is thickly studded with fine, pale-lilac granules, a little bluer than the neutrophile granules. In heavily stained films the granules in the older cells may be quite coarse and bluish. The margins of the cell are thin and often irregular. The nucleus in the older cells is kidney-shaped or horse-shoe shaped, and often shows small, irregular lobulations along the concave margin (transitionals of Ehrlich). The younger forms have round or oval nuclei (large mononuclears of Ehrlich). The surface of the nucleus often shows several deep creases which give it a coarsely convoluted appearance. The nucleus takes a pale bluish violet stain, and contains a characteristically reticulated chromatin network with slight thickenings at the intersections of the threads. In the younger cells it has a fine, spongy texture which may be compared with that of fine lace, whereas in the older cells it is coarser and stains more intensely. There are no nucleoli. Monocytes show amoeboid motility and are actively phagocytic.

Monoblasts, the primitive monocytes, differ in having a more basophilic cytoplasm and sparse granulations. The nucleus is fine in texture and may contain a few small, pale-blue nucleoli.

Pathological leukocytes are cells which, in some respect, differ markedly in their appearance from normal cells of their type. The difference may result from a developmental anomaly in conditions in which blood cell formation is disturbed. There may be an abnormality in the size of the cell, in the quantity or staining properties of the cytoplasm or its granules, or in the size, the staining, or the degree of segmentation of the

nucleus. Pathological lymphocytes occur frequently in infections, and do not necessarily suggest leukaemia.

Pathological changes may also occur as a result of toxic injury to the cells in acute infections. Such cells may show vacuoles in the cytoplasm or the nucleus. The margins of the cell may be irregular or "moth eaten." The neutrophilic granules may be sparse or absent, or may stain abnormally. The presence of coarse bluish granules has been emphasized and also bluish staining areas in the cytoplasm. The nucleus may be swollen and show loss of its finer structure, or it may be pycnotic. If marked in degree, such changes have a bad prognostic significance.

Smudged cells, if not due to poor technique in making the film, suggest the presence of large, fragile cells. In certain cases of leukaemia these may be numerous, and have been termed "basket" cells. Even in normal blood many of the neutrophiles at times may be smudged. Sabin believes that these smudges represent showers of disintegrating leukocytes, because she finds numerous non-motile leukocytes in supravitally stained films made at the same time.

#### ORIGIN OF THE BLOOD CELLS

The primitive precursors of the blood cells are the cells of the reticulo-endothelial system. These cells are distinguished physiologically by their capacity to engulf and store particulate matter, such as India ink, or fragments of red cells; or colloidal dyes, such as pyrrol blue. These cells comprise: (1) reticulum cells, irregularly stellate in shape, scattered throughout the body in the connective tissue about the blood vessels. According to Corner these cells can be distinguished by demonstrating the presence of reticulum fibrils within the substance of the cell by special staining methods (disputed). (2) The specialized endothelial cells of the capillaries and sinuses of certain limited regions of the body: the bone marrow, spleen, liver, lymph follicles, hypophysis, adrenal cortex. (3) The macrophages, also known as wandering cells, clasmatocytes, histiocytes, and endotheliocytes (and probably the monocytes), which are derived from either or both of the preceding types of cells.

The primitive red cells are derived from vascular endothelium. In the early embryo the process of red cell production is widely distributed, but it soon becomes largely concentrated in the liver, and later in the spleen. At birth it is largely confined to the red bone marrow, all bones participating. After about the 16th year it has disappeared from the long bones, and in the normal adult it is largely confined to the vertebrae, ribs, sternum, skull, and pelvis. In response to the stimulus of an anaemia, red cell formation is often resumed in the long bones, occasionally in the spleen, and rarely even in the liver.

According to Doan, in the adult the red cells are normally produced by intravascular proliferation of the endothelium of intersinusoidal capillaries in the marrow. These capillaries are believed usually to be shut off (functionally) from the general circulation but open occasionally to discharge red cells as these mature. The bulk of the erythropoietic tissue is made up of the cells described as "immature normoblasts." Some of these continue in a state of active proliferation, while others develop by a process of maturation into mature red cells. In certain severe anaemias, particularly in pernicious anaemia, these cells are replaced by more primitive types of crythroblasts, the macroblasts or megaloblasts.

The ultimate ancestors of the granular leukocytes are believed to be primitive reticulum cells. In the embryo the leukopoietic tissue has much the same distribution as noted

## ORIGIN OF THE BLOOD CELLS

# Reticulo-endothelial system

Maturation leads	Ex	travascular ori	gin	Intravascu- lar origin	Intra- and extravascu- lar Origin		
Primitive fixed cells		Reticulum cell multipotential		Endoth (duopo			
Primitive free cells	(	Primitive cell multipotential	)				
Differentiating mechanism environ- ment or spe- cific stimulus	In bone marrow	In Connective tissue Spleen Lymph nodes	Inlym- phatic tis- sues	In intersi- nusoidal capillaries of bone marrow (Doan)	All other endothelium		
Unipotential blood cells	Myeloblast	Monoblast	Lympho blast	Megalo- blast Erythro- blast	Clasmato- cyte (histio- cyte)		
Young cells	Myelocyte	Premono- cyte	Young lym- phocyte	Normoblast	(Matura- tion not de- scribed)		
Mature cells	Poly	Monocyte	Mature lympho- cyte	Reticulocyte Red cell			
Degenerating cells	Non-motile (Sabin)		Old lympho- cyte	Fragmented red cells			

This table summarizes the views of those haematologists who believe in the independent origin of each of the types of blood cells ("polyphyletic school"). Those belonging to the "unitarian school" believe that the common ancestor of the different types of cells ("primitive cell" in this table) is a lymphocyte, and that transformation of one type of cell into another may occur, even up to the mature stage. (After Wiseman, Journal of the American Medical Association, Nov. 17, 1934-)

in the case of the red cells. In the normal adult their formation is largely limited to the marrow of the flat bones. When their proliferation is strongly stimulated, as in leukaemia, the distribution becomes wide spread, involving not only the spleen and liver, but also the lymph glands, and sometimes the skin and the connective tissue of the kidneys and other organs, particularly about the blood vessels. Normally the proliferating tissue is largely composed of differentiated myelocytes, but in leukaemia these may be replaced by primitive myelocytes or myeloblasts.

The lymphocytes are formed in the lymph follicles, wherever these may be located. Their immediate precursors are the large cells in the center of the follicles. Their more primitive precursors, the lymphoblasts, are probably derived from reticulum cells.

The monocytes, according to Sabin and many others, are derived from reticulum cells, largely but not exclusively in the spleen. Naegeli and some others believe that they arise in the bone marrow, from myeloblasts or closely related cells. The consensus of opinion now is that they constitute a third type of leukocytes, distinct from both the lymphocytes and the granulocytes. The exact relationship of the monocytes to the histiocytes, or clasmatocytes, is still in dispute. Sabin regards the latter as a distinct type of cell derived from endothelium. There is strong evidence that under special conditions, at least, histiocytes are also derived from monocytes.

There is still disagreement as to many of the details of the process of blood cell formation, particularly as to the relation of the primitive types of cells to one another. There is much to support the view that even in the adult there are primitive (reticulum) "stem" cells which under certain conditions may give rise to any one of the three main types of leukocytes. In leukaemias of any type there is widespread proliferation of the corresponding type of cell. which may be found practically wherever reticulum cells occur. On the other hand there is no convincing evidence of the transformation of one type of leukocyte into another after definite differentiation has occurred. The diagram from Wiseman summarizes one of the more widely accepted theories as to these relationships.

The method of removal of the erythrocytes.—Under normal conditions the average life of the red cells is probably about 4 weeks. Hence the cells from about 125 cc. of blood are removed and replaced daily. Red cells are not normally haemolyzed in the circulation. It is probable (Rous and Robertson) that as a result of the buffetings the cells receive in the circulation, they are gradually broken to pieces without loss of haemoglobin to the plasma. The fragments are ingested by the phagocytic cells of the reticuloendothelial system, largely, but not exclusively, in the spleen. The iron is split off and stored in the liver and spleen. The balance of the haemoglobin molecule is split up, and part is converted into bilirubin in these cells, which secrete it into the blood. It is then taken up by the glandular cells of the liver, and largely excreted in the bile. The bilirubin is converted into urobilin in the intestine and eliminated in the faeces. According to most investigators a considerable part of the bile pigments is reabsorbed from the intestine, and either utilized in the formation of new haemoglobin, or converted into urobilinogen and excreted in the urine. Whipple, however, disputes this, and regards the bile pigments as purely waste products.

#### THE NORMAL DIFFERENTIAL COUNT

The average figures for the normal adult are given in the following table.

	In percentage				Absolute number per cmm.			
	Aver- age, %	Us	ual 1	range,	Aver- age	Usual	range	
Neutrophiles: total	66	50	to	70		3000 tu	6000	
Segmented nuclei	62	50	to	ó.5	200	3000 to	6000	
Nonsegmented nuclei	4	2	to	8	300	200 to	700	
Metamyelocytes	0	0.	ťο	0				
Eosinophiles	1.5	ο.	5 to	3	100	50 to	300	
Basophiles	0.5	0	to	0	25	o to	75	
Lymphocytes: total	20	20	to	30	800	1000 to	3000	
Large	1	0	to	6	100	o to	600	
Small	25	20	to	30	800	1000 to	3000	
Monocytes	6	.3	to	8	4.50	300 to	hoo	
Total leucocyte count					000	5000 to	10,000	

In infants after the first few days the lymphocytes constitute 40% to 60% of the total count, and the neutrophiles are correspondingly reduce 1(30% to 50%). The average adult formula is usually attained at about 5 years of age, but this is quite variable (2 to 10 years or more).

#### LEUKOCYTOSIS

The term leukocytosis signifies an increase in the total leukocyte count (above 10,000). Since in most cases this is due to an increase in the neutrophiles, it is often used inexactly to indicate a neutrophilic leukocytosis. Such a leukocytosis is called relative if the total leukocyte count is within normal limits, but the percentage of neutrophiles is increased (above 70%). It is absolute if the total number is increased, as well as the relative number. A relative neutrophilic leukocytosis usually has about the same significance as a slight absolute leukocytosis.

Occurrence.—Normal individuals may show a neutrophilic leukocytosis:

- 1. After muscular activity (sometimes marked).
- 2. After cold baths (a rapid redistribution of cells in the circulation).
- A digestive leukocytosis has often been described, but recent work has created grave doubt as to its occurrence.
  - 4. In infants during the first few days.
- 5. In pregnancy during the last few weeks (slight), and in the puerperium (sometimes marked, up to 20,000).
  - It occurs in the following pathological conditions:
- 1. In most acute infections; particularly acute pyogenic infections, intra-abdominal inflammations, general sepsis, pyelonephritis, tonsillitis, scarlet fever, acute rheumatic

fever, pneumonia of all types, and meningitis. The total leukocyte count in these conditions usually ranges from 12,000 to 20,000; in lobar pneumonia it often reaches 30,000 to 40,000. It may rarely rise to 100,000.

- 2. In intoxications; as with illuminating gas, lead (up to 20,000 in lead colic), after such drugs as turpentine, acetanilid, potassium chlorate, phenyl hydrazine, salvarsan; in acute gout; and in uraemia.
  - 3. After acute haemorrhage.
- 4. Malignant neoplasms, especially rapidly growing tumors of the liver, gastro-intestinal tract, and bone marrow (metastatic).
- 5. After operations, for 12 to 36 hours, particularly if there has been much tissue injury.
  - 6. Myelogenous leukaemia.

Significance.—In general, a leukocytosis may be regarded as a response of the bone marrow to an increased demand for leukocytes. The degree and type of response vary (1) with the intensity of the stimulus (which depends largely on the species of the infecting organism, on its virulence, and on the location and extent of the area involved), and (2) on the reacting power of the individual. A mild infection may call forth an insignificant response. If the infection is overwhelming, or if the reacting power of the marrow is seriously impaired, there may be no leukocytosis, but even a leukopenia. In an individual with adequate reacting power, the degree of leukocytosis is very roughly proportional to the severity of the infection. In interpreting the leukocytic response to an infection, however, changes in the differential count and qualitative alterations in the leukocytes are more significant than an increase in their total number.

A great deal of work has been devoted to this problem, in an attempt to find some characteristic change which would have prognostic significance in infections, and which might serve as an indication for immediate operation in such conditions as acute appendicitis. The earlier studies emphasized the significance of a rise in the percentage of neutrophiles, and the relation of this rise to the increase in the total leukocyte count. Thus Sondern regarded the latter as a measure of the resistance of the individual, whereas the degree of the increase in the percentage of neutrophiles measured the severity of the infection, or the "degree of toxic absorption." Gibsom devised a chart, and Wilson a formula to express this numerically.

Index of resistance = 
$$(T - 10) - (P - 70)$$
.

T = the total leukocyte count in thousands.

P = percentage of neutrophiles.

A negative index indicates a low resistance and a poor prognosis.

In general, if a blood stream invasion or a specific acute infection (like pneumonia) can be excluded, a neutrophile percentage of 85 or over points to pus formation or gangrene, while a percentage under 80 is against this. Individual exceptions occur, however, particularly in children, and in patients with such infections as typhoid fever or tuberculosis.

More reliable information is obtained by a study of the neutrophiles from the standpoint of their maturity. The available reserve supply of preformed leukocytes is relatively small, and an increased demand must be met largely by the production of new cells by the bone marrow. When hastily produced in response to an infection, many of the cells are hurried into the circulation before development is completed. Arneth was the first to emphasize the significance of this phenomenon, and he attempted to measure it quantitatively by utilizing the degree of segmentation of the nucleus as a criterion of maturity. In making differential counts he divided the neutrophiles into 5 groups. In the first group he included all cells with unsegmented nuclei, and in the second to the fifth groups (inclusive) he put cells with nuclei composed of 2 to 5 lobes respectively. He gave the following figures as the average distribution in normal blood: 5%, 35%, 41%, 17%, 2%. He calculated a numerical "index" by adding together the percentage of cells in the first two groups, and half the figure for the third group (normal index 60). In cases of active infection the relative number of cells in the first two groups was increased, and as these happened to be recorded on the left hand side of his tally sheets, this change was termed a "shift to the left." He regarded this shift as far more significant than a rise in the total leukocyte count. However, grave objections, both theoretical and practical, have been raised to Arneth's counts, and they have been practically abandoned as a clinical procedure.

Several other investigators have suggested simplified procedures to estimate the degree of immaturity of the leukocytes. Of these we shall give only that of Schilling, which is the one most frequently employed. This is best understood by a study of the following illustrative counts, selected from his book. He subdivides the neutrophiles

ILLUSTRATIVE DIFFERENTIAL COUNTS BY SCHILLING'S METHOD.

COMPILED FROM SCHILLING'S "THE BLOOD PICTURE"

				ocyte					
				,	Non-segm neutropl (Stabzell				
Normal Normal range					4	63	23 21-35	6	6,000
Normal range					3-5		21-35	4-8	5,000- 8,000
Appendicitis (mild) Appendicitis:									0,000
(1) Wound infection	1	3	0	2	9	65	()	11	000,51
(2) Peritonitis	0	0	2	2 [	29	41	2	. 5	33,000
(3) Agonal	0	0	12	29	38	21	0	U	25,000
Pneumonia (favorable)	0	0	0	4	32	51	12	I	12,000
Pneumonia (senile, fatal)	0	0	τ	30	18	35	II	5	8,600
Bacterial endocarditis	0	2	0	2	20	53	17	6	5,000
Tuberculosis									
(1) Mild, active	1	2	0	0	3	39	39	16	Low
(2) Advanced	0	0	0	6	39	17	13	25	High
Malaria (MT) paroxysm	0	0	0	22	28	20	20	10	High
Malaria (BT) afebrile period	0	2	0	7	14	46	15	16	6,000
Typhoid fever	0	0	0	0	30	20	38	12	3,000

into four classes, which have already been described. Good, thin films are indispensable for these counts. The differentiation of segmented from non-segmented nuclei is in part a subjective matter, and the examiner should establish his own standard of normal by counts on normal blood.

As a rule in acute infections, particularly those which are accompanied by a neutrophilic leukocytosis, there is an increase in the percentage of cells with non-segmented nuclei (a shift to the left), which is roughly proportional to the severity of the infection.

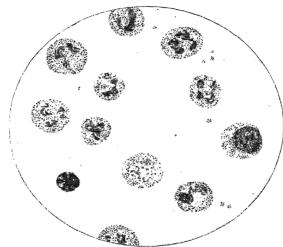


FIG. 72.—Leukocytosis (55,000). Acute lobar pneumonia. Actual field. Marked regenerative "shift to the left." Two myelocytes in field. Lower right: Cell showing toxic-degenerative changes in the granules.

Schilling distinguishes a "regenerative shift," in which "Jugendformen" (metamyelocytes) appear in the blood, from a "degenerative shift," in which the non-segmented cells are "Stabzellen," with coarse, densely stained chromatin. The latter are probably to be regarded as the result of pathological maturation, rather than an indication of simple immaturity. A regenerative shift is the more significant.

In general, the following points indicate a severe infection and usually have an unfavorable prognostic significance.

(1) An extremely high total leukocyte count with a high percentage of neutrophiles.
(2) The failure to develop a leukocytosis, or a leukopenia.
(3) A high percentage of immature cells, especially if they outnumber the mature cells.
(4) Absence of eosinophiles.
(5) A marked reduction in the absolute number of lymphocytes.
(6) Many

cells showing toxic-degenerative changes in the nucleus or cytoplasm. Conversely a favorable significance may be attached to the absence or progressive abatement of these characteristics. Although caution must be used in interpreting the findings in any individual case, such a study undoubtedly gives information of great practical value. A definite trend in either direction as shown by successive examinations is much more significant than the result of a single examination.

Leukopenia.—A notable reduction in the leukocyte count below 5,000 must be mainly at the expense of the neutrophiles. It occurs: (1) Regularly in certain infections, if uncomplicated: typhoid fever, measles, influenza, dengue, kala-azar, and malaria (except during the paroxysms); occasionally in the initial stages of small pox, and in some cases of tuberculosis, especially extensive glandular tuberculosis. (2) Overwhelming infections which are usually associated with a leukocytosis. (3) Malignant neutropenia (agranulocytic angina). (4) Acute or chronic poisoning with such drugs as benzol, acetanilid, sulfanilamide, aminopyrine, lead, mercury, and arsenic (arsphenamine). (5) After excessive radiation. (6) Cachectic or debilitated states. (7) "Exhaustion" of the marrow, as in idiopathic aplastic anaemia, and some cases of leukaemia. (8) Displacement of the leukopoietic tissue of the marrow by other tissue, as in pernícious anaemia, leukaemia, or metastatic neoplasm.

A "simple leukopenia," usually of moderate degree (3000 to 5000), is observed occasionally in otherwise apparently normal individuals. The differential count may be normal, or there may be a slight relative lymphocytosis. There is not necessarily any evident decrease in resistance to ordinary infections.

Eosinophilia occurs chiefly in three groups of conditions: 1. Infection with animal parasites.—In trickinosis it is nearly constant and usually marked (up to 70% of 35,000 cells). It usually occurs in echinococcus and hook worm infections of significant degree, and less regularly with ascaris, taenia, whip worm, strongyloides. etc. In Guam among the natives it is difficult to find an eosinophile count of less than 15%. It is rare in amoebiasis.

- 2. Skin diseases of any type, depending more upon the extent than upon the type of skin involvement.
- Allergic reactions to foreign protein, including bronchial asthma, hay fever. urticaria, angioneurotic oedema, mucous colitis, and serum disease.

It also occurs in (4) chronic myelogenous leukaemia; (5) the acute stage of scarlet fever, and (6) the stage of recovery from many acute infections (in slight degree). It is seen occasionally (7) in Hodgkin's disease (up to 60% in rare instances), (8) with certain tumors of the ovary, (9) in focal lesions of the bone marrow, (10) after splenectomy, and (11) in pernicious anaemia after liver therapy (up to 20%, rarely even to 60%).

Eosinophiles are diminished in the acute phase of most severe infections.

A basophilic leukocytosis occurs commonly in chronic myeloid leukaemia. In other conditions it is infrequent and of no known diagnostic significance.

Lymphocytosis.—A relative lymphocytosis occurs in most of the conditions which show a leukopenia.

An absolute lymphocytosis occurs in certain infections: (1) Whooping cough, including the secondary bronchopneumonias which follow it. A lymphocytosis is nearly constant, and usually marked, from 20,000 to 40,000, rarely to 100,000, with 60% to 80% of normal small lymphocytes. (2) Infectious mononucleosis (glandular fever). (3) Less regularly and less markedly in German measles (with plasma cells), brucella infection, and mumps. (4) In some cases of tuberculosis, chiefly mild or moderately

active cases running a favorable course. (5) In young children, occasionally, in infections which usually cause a neutrophilic leukocytosis. (6) During convalescence from any acute infection. (7) In hyperthyroidism. (8) After exposure to ultraviolet radiation. (9) In lymphatic leukaemia.

The lymphocytes are reduced in absolute number (1) after excessive radiation, (2) during the acute stage of most infections, and (3) in conditions in which the lymphoid tissue is replaced by other tissue, as in myelogenous leukaemia, advanced Hodgkin's disease, and extensive tuberculosis or carcinomatosis of the lymph glands.

Monocytosis occurs: (1) In many cases of malaria and other protozoan infections, including trypanosomiasis, kala azar, and amoebiasis. (2) Less regularly and often only in slight degree in variola, typhus, spotted fever, dengue, yellow fever, measles, and syphilis. (3) In bacterial endocarditis (capillary blood). (4) In active progressive tuberculosis. A reversal of the usual lymphocyte-monocyte ratio suggests a dissemination of tubercles, and has an unfavorable prognostic significance. (5) In Hodgkin's disease. (6) In Banti's disease. (7) In monocytic leukaemia.

A slight increase in monocytes occurs frequently at the height of many acute infections.

Myelocytosis (the presence of myelocytes in the circulating blood) occurs chiefly (1) in myelogenous leukaemia. In usually small numbers myelocytes may appear (2) in any condition associated with a hyperleukocytosis, especially in children. (3) In severe infections or intoxications, regardless of the total leukocyte count, in which there is a marked "shift to the left" (a "leukaemoid reaction"). (4) In pernicious anaemia and other severe anaemias. (5) In focal lesions of the bone marrow, especially metastatic neoplasm.

#### THE BLOOD PLATELETS

The blood platelets, or thrombocytes, are round or oval bodies usually 2 to  $3\mu$  in diameter, although they vary greatly in size and shape. In films they show a marked tendency to form clumps. With Wright's stain they show a faintly bluish background studded with numerous fine, dull purplish-red granules which resemble chromatin. They contain no nucleus. They are believed to arise from the megakaryocytes, the multinucleated giant cells of the bone marrow, as pinched-off fragments of the cytoplasm of these cells. Howell (1937) has demonstrated that in cats they are formed from megakaryocytes lying in the pulmonary capillaries rather than in the bone marrow. In pathological conditions, particularly in chronic myelogenous leukaemia, occasionally in Hodgkin's disease and pernicious anaemia, large masses of cytoplasm may be found in blood films.

In these conditions megakaryocyte nuclei are occasionally found in the blood. The typical, large lobate nuclei seen in marrow films can not pass through the pulmonary capillaries, but small nuclei or nuclear fragments may get through. They are 2 to 4 times the diameter of a red cell or larger, round, oval, or irregular in shape, and take an intense, reddish-purple stain. The chromatin tends to be condensed slightly in coarse splotches or irregular bands separated by somewhat paler staining areas, giving the nucleus an irregularly striped or coarsely spotty appearance. The nuclei may be naked, or there may be small, ragged fringes of platelet material attached.

Platelets are indispensable for normal blood coagulation. Their life is brief, about 3 or 4 days. They are thought to be removed by the spleen and other reticuloendo-

thelial tissues. The stated normal number varies with the method used to count them (see p. 300).

Platelets are increased in most conditions associated with active cell formation in the marrow. Among these are: (1) Many acute infections. (2) After haemorrhage. (3) Chronic myelogenous leukaemia. (4) Polycythaemia. (5) Hodgkin's disease.

Platelets are diminished in: (1) Idiopathic purpura haemorrhagica. (2) Pernicious anaemia. (3) Aplastic anaemias. (4) Acute leukaemias. (The "primary" thrombocytopenias). (5) Some severe infections. (6) Acute or chronic poisoning with benzol, radium, arsphenamine, gold salts, etc. (7) Excessive radiation. (Secondary thrombocytopenias).

### CHAPTER XV

# DISEASES OF THE BLOOD

# DISEASES INVOLVING PRIMARILY THE RED CELLS

### THE ANAEMIAS

By anaemia is meant a reduction below the normal in the amount of haemoglobin (and usually in the number and volume of the red cells) per unit volume of blood. It is a symptom of a great variety of unrelated diseases.

There is no single scheme of classification of the anaemias which is comprehensive and satisfactory. The most logical method is based on the pathogenesis of the anaemia. (1) Those due to loss of blood, the posthaemorrhagic anaemias. (2) Those due to inadequate blood formation, the deficiency anaemias and the aplastic anaemias. (3) Those due to increased blood destruction, the haemolytic anaemias. There are practical difficulties in the application of this classification, because in many cases inadequate blood formation and increased blood destruction both play an important rôle. Nevertheless we have utilized this method as far as practicable in the following discussion.

From the morphological standpoint the anaemias may be classified according to the mean corpuscular haemoglobin or color index as hyperchromic, normochromic, or hypochromic; and according to the mean corpuscular volume or volume index as macrocytic (megalocytic), normocytic, or microcytic. This classification has practical significance, since, in general, macrocytic anaemias are benefited by liver or liver extract, whereas the hypochromic anaemias are helped by iron. The terms, however, should be regarded as descriptive, and not as diagnostic. The terms "normocytic" and "normochromic" should be understood to mean merely that the average volume or haemoglobin content of the red cells is within normal limits. Otherwise they may be misleading, since the cells may be abnormal in other respects, and individual cells may be markedly abnormal in size and color.

The current tendency is to drop the use of the old terms "primary anaemia," supposedly due to some inherent disease of the blood-forming tissues; and "secondary anaemia," due to some obvious extraneous cause.

# A. Anaemias Due to Loss of Blood

These anaemias are hypochromic and usually microcytic.

1. Acute Posthaemorrhagic Anaemia.—The danger to life from a single profuse haemorrhage is from circulatory failure (shock) due to the

lack of a sufficient volume of blood to fill the vessels, and not from a deficiency of haemoglobin. The first step in regeneration after an acute haemorrhage is the restoration of plasma volume, by the passage of tissue fluids into the vessels. This results in a dilution of the blood, with a gradual fall in red cell count and haemoglobin, which is not complete until from 12 to 24 hours after bleeding has ceased. The resulting anaemia stimulates a rapid production of new red cells which tend to be imperfectly formed, to be inadequately supplied with haemoglobin, and to be hurried into the circulation before development (maturation) is completed. Within 24 to 48 hours there appear red cells which are smaller than the average, and which are pale. The color index and volume index fall. The reticulocytes are increased, polychromatophilic cells appear, and occasionally a few normoblasts. There is usually a neutrophilic leukocytosis and an increase in platelets. With the influx of new cells there is a gradual rise in the red cell count, and (more gradual) in haemoglobin. The maximum abnormalities in the cells, however, are not reached until about the eighth or tenth day. In otherwise normal individuals completely normal conditions are restored after about 30 days.

2. Chronic Posthaemorrhagic Anaemia.—The changes in the blood are usually similar to those described above, but they tend to become more marked. In severe cases there is a marked degree of anisocytosis, a majority of the red cells are smaller than normal in diameter and volume, microcytes are numerous, and some poikilocytes may be present. As the iron stores of the body become depleted the concentration of haemoglobin in the cells diminishes, the color index and the saturation index fall, and the cells are pale. The centers may be colorless, so that the cells look like rings (pessary forms). There is no increase in the bilirubin in the serum. This is the typical picture of a hypochromic microcytic anaemia. The degree of anaemia may be severe. Counts of 2 million red cells and 20% of haemoglobin are not unusual, and rarely they may fall to half these figures.

The bone marrow is hyperplastic, and the predominant cells are normoblasts. The fatty marrow of the tibia and other long bones is often replaced by such red hyperplastic marrow.

While active formation of red cells continues, immature cells will be present. In cases with protracted bleeding, however, the marrow may become exhausted (aplastic). In such cases the immature red cells disappear, and there may be a reduction in the number of leukocytes and platelets. In exceptional cases of this type, particularly those with long continued small haemorrhages, the few cells which are formed may be more nearly normal, and the color index may approach 1.0.

Identical changes are met with in hookworm infection.

# B. Anaemias Due to Inadequate Blood Formation

# a. Anaemias Related to a Deficiency of Iron

These anaemias are hypochromic, and usually microcytic in type. They include: (1) Anaemias due to *lack of iron* in the diet, seen most frequently in infants and young children on a diet consisting largely or exclusively of milk. The anaemia appears earlier and is more severe in children of anaemic mothers, because of inadequate storage of iron in the foetal tissues.

- (2) Anaemias due to faulty absorption of iron from the digestive tract, as in chronic diarrhoea, colitis, and some cases of sprue and indiopathic steatorrhoea.
- (3) Anaemias due to loss of iron by external haemorrhage (already discussed).
  - (4) Anaemia in severe hookworm infection.
  - (5) Anaemia in some cases of cancer of the stomach.
  - (6) Anaemia in some cases after extensive operations on the stomach.
  - (7) Idiopathic hypochromic anaemia.
  - (8) Chlorosis.

Idiopathic hypochromic anaemia (primary microcytic anaemia, simple achlorhydric anaemia, chronic chlorosis) is a chronic disease largely (95%) limited to women, chiefly those between 20 and 50 years of age. Clinically it is characterized by an insidious onset, by the gradual development of marked weakness, lethargy, and nervous instability; and by digestive discomforts; gaseous distension, epigastric pain, occasionally diarrhoea and anorexia, or a fickle appetite. This often leads the patients to avoid meats, fruits, and green vegetables, foods rich in iron, and tends to aggravate the anaemia. Marked loss of weight is exceptional.

Soreness of the tongue and mouth is common. There is a glossitis and stomatitis which leads to atrophy of the mucous membrane. In at least half the cases there is atrophy of the papillae about the tip and margins of the tongue. The process often extends to the dorsum of the tongue, which becomes smooth and polished in appearance; and to the lips, which may show cracks and fissures about the corners of the mouth. In a small group of cases (Plummer-Vinson syndrome, anaemia with dysphagia) it extends into the pharynx and hypopharynx, causing dysphagia, which is attributed to reflex spasm of the inferior constrictor.

In about half the cases the nails become tender, thin, and brittle, they tend to loosen from the nail bed, and may become flattened or even concave and spoon shaped on the dorsal surface (koilonychia).

Paraesthesias of the extremities are common, as in pernicious anaemia, but combined sclerosis of the cord does not occur.

Menorrhagia is a common symptom, and the anaemia may erroneously be attributed simply to the loss of blood. Otherwise there is rarely any abnormal tendency to bleed, or any disturbance of coagulation. Fertility is but little affected.

The spleen is enlarged in about 40% of the cases.

The *skin* becomes inelastic and wrinkled, it may be waxy white or show slight brownish pigmentation. The sclerae are bluish white. There is never jaundice.

The gastric juice shows a hypochlorhydria or an achlorhydria in at least 85% of the cases, and a complete achlorhydria after histamine in about 60%. Mucus is abundant. The ordinary ferments are often

diminished or absent, but the in-

The blood shows all the features characteristic of a hypochromic microcytic anaemia, as described in posthaemorrhagic anaemia. In the average patient the red cell count is between 3.5 and 4.0 million, the haemoglobin 6 to 8 Gm, or 40 to 50%. In severe cathey may fall to 1.5 million and 2.0 Gm. The striking feature is the extreme pallor of the cells, and the degree of

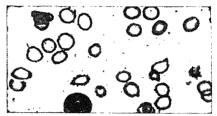


FIG. 73.—Red blood corpuscles showing deficient haemoglobin (achromia). From a well marked case of chlorosis. Wright's stain (×750.) (J. C. Todd, "Clinical Diagnosis.")

reduction in the color index (0.3) and in the mean haemoglobin content  $(11\gamma\gamma)$  and haemoglobin concentration (22%) in the red cells which may develop. The leukocytes and platelets are usually normal.

In untreated cases reticulocytes are sparse. However, biopsy shows the bone marrow to be markedly hyperplastic (normoblastic). There appears to be some obstacle to the maturation of the cells. An effective stimulus to their maturation and delivery into the circulation is provided by the administration (by mouth) of large doses of iron (6 Gm. or more per day of ferri et ammonii citras, or correspondingly large doses, in terms of their metallic iron content, of other preparations). In severe cases within 3 or 4 days after an adequate dose of iron is started, there is a rise in reticulocytes which reaches a peak on the seventh or eighth day, and which is roughly inversely proportional to the haemoglobin percentage. A satisfactory response is indicated by a rise in reticulocytes to 15% if the Hb. is 20%; to 8%, if the Hb. is 40%; and to 5%, if the Hb. is 60%; and by an average daily rise in Hb. of at least 1% (in some cases 2%). An equally striking rise may be obtained in severe hypochromic anaemia of other types, as in hookworm anaemia (Castle and Rhodes, 1932), and even

(temporarily) in cancer of the stomach. There is prompt relief (not always complete) of the symptoms and subsidence of the physical signs, except that the lingual atrophy and the achlorhydria persist. Liver extracts are ineffective.

The disease is rarely directly fatal, even if untreated, but it often causes a protracted and profound degree of chronic invalidism. There is little tendency to spontaneous remission, until after the menopause. An exacerbation may be precipitated by a pregnancy, or by intercurrent infections.

The etiology is still obscure. A major factor is deficient iron absorption resulting from the defective gastric secretion. In some cases the latter is attributable to an inherent constitutional (sometimes familial) defect. In some it may be due to a chronic gastritis. A severe anaemia of any type, however, may cause a temporary disappearance of free HCl from the gastric juice. An identical condition has been described in patients (male as well as female) after gastroenterostomy, and after extensive gastric resection, and in some cases of cancer of the stomach. Other contributing causes are an inadequate, ill-balanced diet, and the drain on the iron reserves resulting from menstruation and pregnancy. The defect is permanent, since relapse occurs if the administration of iron is stopped.

Clinically this disease resembles pernicious anaemia in many ways, although the changes in the blood are entirely different. They are similar in that both apparently depend on (different) deficiencies of gastric secretion, and both occasionally are familial. In several families idiopathic hypochromic anaemia has developed in females and pernicious anaemia in males, and rarely both diseases have developed successively in the same individual. The possibility of a double deficiency must be remembered, although outspoken examples of this are surprisingly rare.

Chlorosis is described as a disease of unknown etiology, limited to females and occurring chiefly during adolescence, characterized by the development of an anaemia of the hypochromic type, and by a prompt response to iron medication. The blood changes are identical with those in idiopathic hypochromic anaemia. The chief clinical differences are: (1) the younger age incidence in chlorosis, which usually subsides spontaneously at the age at which idiopathic hypochromic anaemia is most frequent; (2) normal or excessive amounts of HCl in the gastric juice in chlorosis, and (3) the response to smaller doses of iron. It is probable that chlorosis is due simply to an extreme lack of iron in the diet. Chlorosis has become rare in all countries and has practically disappeared in the United States.

Copper.—In animals (cattle, rats, swine) a diet made grossly deficient in copper as well as in iron produces an anaemia which does not respond to the administration of iron alone, but which improves promptly if copper is also given. A minute amount of copper is apparently necessary for the utilization of iron. It is very doubtful, however, whether a significant deficiency in copper occurs naturally in man, even on poor diets, except possibly in young children.

# b. Anaemias Related to a Deficiency of the Anti-anaemic Factor in Liver

These anaemias are macrocytic, and usually hyperchromic in type. They include: (r) Primary pernicious anaemia (by far the most frequent and important).

Some cases of: (2) Sprue, idiopathic steatorrhoea, and other chronic intestinal disturbances. (Fistulae, multiple anastomoses, chronic obstruction).

- (3) Diphyllobothrium latum (fish tapeworm) infection.
- (4) Cancer of the stomach (rare).
- (5) Complete resection of the stomach.
- (6) Chronic diseases of the liver.
- (7) Pregnancy (rarely).
- (8) Tropical megalocytic anaemia.

Recent work by Minot, Castle, and many others has shown that the normal development and maturation of red cells is dependent on the activity of a specific substance which is commonly called the anti-anaemic principle of liver, or potent substance. The production of this antianaemic principle depends on the interaction of two other substances: (1) An extrinsic factor which is furnished by the diet, is abundant in muscle and in yeast, and for a time was erroneously thought to be identical with vitamin B2; and (2) an intrinsic factor, which is present in normal gastric juice (possibly secreted also in the proximal part of the duodenum), and presumably is a ferment, although it is not identical with any of the previously recognized gastric ferments. The effective utilization of this anti-anaemic principle depends also (3) on adequate absorption from the gastrointestinal tract, and probably (4) on its storage by the liver, and presumably on its orderly release by the liver and distribution to the haemopoietic tissues as it is needed. A disturbance of any one of these functions, whatever the cause, tends to produce an anaemia which is macrocytic and hyperchromic in type. A macrocytic anaemia may occur, however, in diseases which are not associated with a disturbance of the anti-pernicious anaemia principle (leukaemia, primary aplastic anaemia. etc.).

In pernicious anaemia the disturbance is due to a partial or complete lack of the intrinsic factor in the gastric juice. In sprue and allied conditions deficient absorption is probably the usual cause. In chronic liver disease impaired capacity to store the material may be at fault. In some cases, as in certain tropical anaemias, there may be a lack of the extrinsic factor in the diet. In pregnancy there is probably an increased demand together with a relatively inadequate formation of the substance. Any of these anaemias is usually relieved by supplying adequate quantities of the active material, preformed, as by feeding liver, or by injecting parenterally suitable extracts of liver. Various British investigators have emphasized the fact that the administration of marmite (an autolyzed extract of brewers' yeast) is as effective as liver in those cases in which the anaemia is due to lack of the extrinsic factor, in many cases of sprue and idiopathic steatorrhoea, and to a limited extent in some cases of pernicious anaemia.

The active principle as it is obtained from liver differs in its thermostability and in other properties from that present within the gastrointestinal tract ("addisin," "haemopoietin"), and in the stomach tissue ("ventriculin"). Where this elaboration occurs is not known. That the liver serves as a storehouse for the substance seems certain. It has been demonstrated in the liver of patients dying of unrelated diseases by injecting suitable extracts of such livers (autopsy material) into patients with pernicious anaemia and observing a reticulocyte crisis, but it is absent in the liver of patients dying of untreated pernicious anaemia. It also disappears from the otherwise normal livers of gastrectomized swine, and of swine in which sprue has been experimentally produced.

Although the changes in the blood are usually more characteristic and more marked in degree in untreated cases of pernicious anaemia than in the other conditions mentioned, in some cases of the latter (e.g. sprue, fish tapeworm infection) they may be indistinguishable. In all these conditions the bone marrow shows megaloblastic hyperplasia in varying degree.

Pernicious anaemia is characterized clinically by an insidious onset, usually in adults of middle age; by the gradual development of a severe anaemia, with weakness, dyspnoea, and other symptoms of haemoglobin deficiency; by a protracted course, marked by remissions and exacerbations; and (in untreated cases) by a fatal termination.

During the active periods of the disease digestive disturbances are common: anorexia, gaseous distension, epigastric discomfort, sometimes nausea and vomiting, or diarrhoea, and occasionally crises of sharp colicky abdominal pain. Sore mouth and sore tongue due to a stomatitis and glossitis are common complaints. There is nearly always atrophy of the papillae of the torque, which presents an abnormally clean, smooth,

active stages there is fever, associated with evide

Paraesthesias of the extremities nearly always develop, and are often an early symptom. Focal degenerations of the cord (combined sclerosis) are common. They occur (1) in the posterior columns, causing ataxia, weakness, and minor sensory disturbances, particularly loss of the vibratory sense over the lower legs, diminished reflexes, occasionally hyperaesthesias; (2) in the lateral columns, causing spasticity, exaggerated reflexes, and less often sphincter disturbances. These changes are not proportional to the anaemia, and may antedate it. Rarely combined sclerosis occurs in patients who do not develop an anaemia, although as in typical pernicious anaemia they show an achlorhydria; and usually some degree of macrocytosis. Combined sclerosis is extremely rare in the other related anaemias. Peripheral neuritis also occurs, and may account for the paraesthesias in some cases. Minor cerebral disturbances are common.

In practically all cases there is an achlorhydria, even after histamine injection. The volume secreted is scanty and is increased but little by histamine. Mucus is scanty. The ordinary ferments are usually diminished, and often absent (true achylia). The gastric deficiency is permanent, even in well treated patients. A few rare cases have been reported, who showed free HCl in the gastric juice. The intrinsic factor

has been absent in those cases of this group which have been tested as to this point. There is some evidence to indicate that the lack of intrinsic factor in pernicious anaemia, in some cases at least, is relative rather than absolute. It has been suggested, without as yet definite proof, that variations in the amount of intrinsic factor secreted may account for the fluctuations in the course of the disease

The blood shows a marked reduction in the red cell count, frequently to 2.0, rarely to 0.5 million or less. The haemoglobin is relatively less reduced, so that the color index and mean haemoglobin content of the cells is increased. The volume index and mean corpuscular volume are also increased, more regularly and often more markedly than is the color index. The haemoglobin concentration in the cells is normal or slightly reduced. Anisocytosis is marked, and in severe cases it becomes more

pronounced than in any other anaemia. Poikilocytes, microcytes, and macrocytes are numerous. Large, oval, deeply staining cells are highly characteristic, and a few are usually present in the early stages of the disease and during the remissions. The mean diameter is increased (to 8.5 to  $o\mu$ ), and the cells are dark (the thickness is also increased).

A few normoblasts are present in most of the cases with marked anaemia. Typical megaloblasts are present at some stage of the disease in untreated  $\frac{r_{10}}{M.m}$ , Megaloblasts: n, normoblast; s, patients, but they may be hard to stippling (punctuate basophilia). find. They can rarely be found after

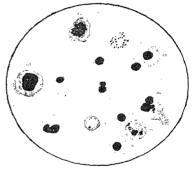


Fig. 74.—Pernicious anaemia.

treatment with liver, even though this is inadequate in quantity. During the blood crises which occasionally occur in untreated patients, there is a sudden outpouring of normoblasts and megaloblasts, reticulocytes, polychromatophilic cells, and cells with nuclear particles.

The platelets are reduced. There is a leukopenia. A few myelocytes are often present, but on the other hand there is a "shift to the right" in the sense of Arneth, with occasional huge neutrophiles containing hypersegmented nuclei with 6 to 10 lobes, "pernicious anaemia neutrophiles."

During the exacerbations of the disease there is mild jaundice, an increase in the bilirubin in the serum, and all the other characteristic features of a haemolytic anaemia. It is generally believed, however, that this increased blood destruction is not a primary cause of the anaemia, but is the result of the entrance into the circulation of imperfectly formed cells which fall ready victims to the normal physiological processes for the removal of defective cells.

In patients with a red cell count below 3.5 million adequate liver therapy is followed by a transient *reticulocyte crisis*, which begins on about the third day, and reaches a peak on about the 7th or 8th day. The height of the peak varies inversely with that of the initial red cell count.

With a red cell count of 1.0 million the reticulocytes should reach 35 to 40%; with 2.0, about 20%; and with 3.0, about 5%. A lesser rise indicates either a mistaken diagnosis, inadequate dosage, or some complicating disease. If the initial dose of active principle has been too small, as indicated by an inadequate reticulocyte response, an increase in the dose will be followed by a second reticulocyte crisis. However, if the initial dose was adequate, a further increase will have no significant effect on the reticulocytes. These observations have proved to be of great practical value in the control of treatment. In favorable cases the red cell count may rise 2.0 million in one month, normal figures will be attained after 2 to 3 months, and the qualitative abnormalities entirely disappear except for the persistence of a few macrocytes. The reappearance of these abnormalities or a fall in the red cell count indicates that the maintenance dose of liver is inadequate. Dried defatted stomach (by mouth) is as effective as liver so administered.

Failure to secure a satisfactory response is often due to deficient absorption from the gastrointestinal tract. In such cases excellent results can be obtained by intramuscular injections, which on the average are at least 50 times as effective as the administration of equivalent doses by mouth. With the best preparations a maximum response follows the daily intramuscular injection of extract from 15 to 20 Gm. of liver. A normal count can usually be maintained by a similar dose given once a week, and in some cases once a month. Patients with combined sclerosis require far larger doses, continued for many months. To control this process it may be necessary to give 2 or 3 times the amount which suffices to restore and maintain a normal red cell count. Improvement at best is slow, and is limited by the degree to which irremediable atrophy of nerve cells has occurred.

Although the pathogenesis of pernicious anaemia is now fairly clear, the underlying cause of the defective gastric secretion remains obscure. In some cases it is a familial constitutional defect. Many families have been reported in which two or more members have had pernicious anaemia, or in which other members have had achylia. Most patients with pernicious anaemia are sthenic in type, and have a light complexion, with fine, often prematurely grey hair. However, the disease may (rarely) occur in negroes.

The theory that a chronic dietary deficiency plays a part is attractive and receives some support from the experiments of Miller and Rhoads (1935). By feeding swine a suitably deficient diet, they produced a diseased state closely resembling tropical sprue and (less closely) pernicious anaemia in man. These animals showed an anaemia (usually macrocytic), with gastrointestinal disturbances and stomatitis, the intrinsic factor disappeared from the gastric juice and the anti-anaemic principle from the liver, and the bone marrow showed megaloblastic hyperplasia. The condition responded to injections of liver extract.

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Tropical megalocytic anaemia is a disease described as occurring in the native population of west Africa, India, and China (Wills and Mehta, 1930). It occurs chiefly in women between 20 and 30, and is often precipitated by pregnancy. The symptoms are those of any severe anaemia. Oedema is often marked. There may be a glossitis, but marked gastrointestinal disturbances are exceptional, and achlorhydria is rare. The blood shows a macrocytic hyperchromic anaemia which may be severe, with marked anisocytosis and many megalocytes, but without an increase in serum bilirubin, and but few poikilocytes and polychromatophilic cells. It is believed to be due purely to dietary deficiency (in extrinsic factor), as it is cured permanently by marmite.

Sprue is a tropical or subtropical disease of unknown etiology occurring most frequently in India, China, and the East Indies, where it is apt to attack white immigrants from temperate latitudes. It also occurs in the West Indies and has been observed in the southern United States. It occurs chiefly in adults, more frequently in women. It is characterized by the gradual development of a chronic morning diarrhoea with progressive emaciation, weakness, and anaemia. The stools are typically bulky, greyish, pultaceous, and frothy, and contain large amounts of fat, chiefly as fatty acids. Glossitis and marked atrophy of the mucous membrane of the tongue and the entire gastrointestinal tract occur, associated with great flatulent distension. Free HCl is present in the gastric juice in most cases, and (if absent) may return during a remission. The intrinsic factor of Castle has been present in some cases and absent in others. There are no bone changes and no gross disturbance of calcium metabolism.

Anaemia is present in most cases, but varies much in severity (red cell count usually about 3.0, but may fall below 1.0). It is usually macrocytic and mildly hyperchromic in type, resembling pernicious anaemia except that the abnormalities are less marked. The anaemia often responds to yeast, and regularly to liver. It is probably due mainly to defective absorption of the anti-anaemic principle, or in some cases, to lack of the intrinsic factor. In a minority of the cases the anaemia is hypochromic, and these cases respond to iron but not to liver.

Idiopathic steatorrhoea (coeliac disease, non-tropical sprue) is a disease of temperate climates which usually begins in infancy or childhood, although it may not be recognized until adult life. It is characterized (1) by chronic diarrhoea, with abdominal distension, and fatty but not frothy stools; (2) by a disturbance of metabolism associated with defective absorption of calcium salts and vitamin D, and characterized by osteoporosis, pains in the bones, bone deformities, and pathological fractures; and (3) by emaciation and anaemia, which are often severe. Achlorhydria is rare. Glossitis may occur, and some degree of atrophy of the lingual papillae is not uncommon. Faecal excretion of calcium is increased, the blood calcium is often low, and latent tetany is common. Lenticular opacities may occur, and occasionally cutaneous eruptions suggestive of pellagra.

The anaemia is usually hypochromic in type, particularly in children. The color index is low, and anisocytosis is marked. The average cell diameter is usually within normal limits, but in some cases it is distinctly increased. Rarely numerous normoblasts have been present. The anaemia (but not the other symptoms) is relieved by iron, and it is attributed to defective absorption of iron.

In other cases, particularly in adults, the anaemia is macrocytic and mildly hyperchromic, resembling that commonly seen in sprue. In this type the anaemia responds to the administration of liver, or large doses of yeast preparations, and is attributed to defective absorption of the anti-anaemic principle.

Diphyllobothrium latum infection causes anaemia in only a very small proportion of the infected individuals. In these cases it may be severe and practically indistinguishable from pernicious anaemia, except that combined sclerosis rarely if ever occurs. The anaemia is usually cured permanently simply by expulsion of the worm. It is also cured by liver, but this must be continued until the worm is expelled. Free HCl is usually absent, but may return after expulsion of the worm. There are no data regarding the presence of the intrinsic factor.

Cancer of the stomach often causes anaemia, which is almost invariably hypochromic and microcytic in type. In the absence of bleeding it is usually moderate in degree, but may be extreme (R.B.C. 1.0, Hb. 15%), and quite like that in idiopathic hypochromic anaemia. In rare instances, which are of theoretical interest but little practical importance, a macrocytic hyperchromic anaemia develops which may be indistinguishable from pernicious anaemia. This may temporarily improve under liver, and is attributed to loss of the intrinsic factor. The anaemia which follows extensive operations on the stomach also is more often hypochromic than hyperchromic in type.

Myxoedema frequently causes an anaemia which may be either hypochromic or hyperchromic in type. The latter type of anaemia is relieved by liver, and is attributed to a lack of intrinsic factor, which may be temporary (relieved by administration of thyroid alone), or permanent (requiring continuous administration of liver, which controls the anaemia, but not the myxoedema).

Pregnancy is so frequently associated with a mild hypochromic type of anaemia that some have regarded this as physiological. This usually increases gradually from the third to the seventh month, after which there may be some spontaneous improvement. The red cell count not infrequently falls to 3.5, and the Hb. to 50 to 60%. Occasionally much lower figures are observed. The anaemia responds well to iron, and is due to an iron deficiency. The latter is probably due in part, at least, to poor absorption, associated with the hypochlorhydria or achlorhydria which is commonly observed during pregnancy. It is partly the result of an increased need for iron to supply the foetal tissues. The apparent degree of the anaemia is somewhat exaggerated by the hydraemia which is

present. The anaemia usually subsides after delivery. Failure to do so suggests the presence of idiopathic hypochromic anaemia, which is markedly aggravated by pregnancy. There is apt to be a recurrence in subsequent pregnancies.

In relatively rare instances a hyperchromic macrocytic anaemia develops. The changes in the blood resemble closely those in pernicious anaemia. There are evidences of increased blood destruction. The disease is severe, runs a relatively acute course, without remissions, and is often fatal if untreated. It may appear during the puerperium, but spontaneous recovery may occur after delivery. It responds well to liver (frequently also to transfusions), and recovery is usually permanent. It may not recur during subsequent pregnancies. It is caused by a lack of the anti-anaemic principle, but whether this is due to inadequate absorption, or to (temporary) lack of intrinsic factor is not established. There is usually free HCl in the gastric juice.

# C. Anaemias Related to a Functional Insufficiency of the Bone Marrow (The Aplastic or Aregeneratory Anaemias)

The inadequacy is usually relative, but in rare instances there appears to be a virtual cessation of red cell formation. The marrow is aplastic in varying degree. Among the more important conditions in which anaemia of this type occasionally occurs are the following:

- 1. In new born infants as a rare constitutional defect.
- 2. As a terminal phenomenon in anaemias of other types, as in chronic posthaemorrhagic anaemia, pernicious anaemia, and the myelophthisic anaemias.
- 3. Anaemias secondary to infection, particularly to such chronic infections as subacute bacterial endocarditis, chronic infectious arthritis, chronic pyogenic infections, and oral sepsis.
  - 4. In advanced chronic nephritis (usually hypochromic in type).
- 5. Malignant disease, in most of those cases in which haemorrhage does not play a part.
- 6. Dietary insufficiencies, other than simple lack of iron, including the avitaminoses, such as scurvy, etc.
- 7. Chronic poisoning with such chemicals as benzol, trinitrotoluol, arsenic, gold or radium salts (Martland, 1931).
  - 8. After excessive exposure to X-rays or radium.
  - 9. Acute idiopathic aplastic anaemia.
  - 10. The myelophthisic anaemias.

This list manifestly includes many heterogeneous unrelated conditions, and the changes in the blood which they show vary in detail. A high grade of anaemia may occur in all of them. In the milder cases some new cell formation persists, and the blood shows imperfectly formed cells and immature red cells in small numbers. The blood often resembles that in cases of posthaemorrhagic anaemia, except that the hypochromia and microcytosis are usually less marked. In severe advanced cases the appearance of the blood is more distinctive. Despite a marked degree of anaemia, the individual red cells which remain are relatively normal. Anisocytosis is slight, and the color index and volume index are usually within normal limits. The anaemia is "normocytic" and "normochromic." Reticulocytes and other immature red cells are entirely absent. There is a neutrophilic leukopenia and a reduction in platelets which may be extreme, and associated with a (symptomatic) purpura haemorrhagica. There is no evidence of increased blood destruction.

In Idiopathic aplastic anaemia these features are seen in maximum degree. This is a rare disease of unknown cause, occurring chiefly in adolescents and young adults, characterized by progressive weakness and prostration, fever, rapidly developing anaemia, extreme leukopenia, purpura and bleeding, and a fatal outcome within a few weeks or months. It is the "aleukia" of German writers. The bone marrow is markedly aplastic. It has been confused with pernicious anaemia, and (more reasonably) with idiopathic purpura and acute leukopenic leukaemia. Cases of severe poisoning with benzol, radium, etc., may present an identical picture.

Scurvy may cause a severe anaemia (R.B.C. 2.0, Hb. 30%) which is hypochromic and usually microcytic in type. It can not be explained by the (relatively trivial) haemorrhages which occur. It is not influenced by iron or liver, but responds to vitamin C with a reticulocyte crisis and rapid improvement.

Myelophthisic anaemia is a term applied to those conditions in which the erythropoietic tissue of the marrow is invaded and more or less crowded out by other tissue. Except in extensive leukaemic infiltration, the bulk of the invading tissue is rarely sufficient quantitatively to explain the anaemia on the basis of simple mechanical displacement, and a functional inadequacy of the remaining marrow must be assumed. These include:

- 1. Osteosclerotic anaemia.
- 2. The leukaemias.
- 3. Some cases of Gaucher's disease, and allied conditions.
- 4. Metastatic tumors in the bone marrow (frequently).
- 5. Multiple myelomata (occasionally).
- 6. Hodgkin's disease (rarely, disputed).

In many cases the anaemia is slight or moderate in degree, but it may be severe. Anisocytosis is often marked, but the color index and volume index are usually about normal or moderately reduced. In many (but not all) cases the blood shows changes suggesting intense stimulation (or irritation) of the marrow. There are numerous immature erythrocytes, including many normoblasts and macroblasts, and even megaloblasts. There is a neutrophilic leukocytosis, with myelocytes in varying number, and occasionally a few myeloblasts. They are sometimes classed as erythroblastic anaemias. (Vaughn). Areas of hyperplastic erythropoietic tissue are commonly found in the long bones, or sometimes in the spleen and other extramedullary tissues.

Osteosclerotic anaemia is a term usually applied to the condition described by Albers-Schönberg as "marble bone" disease. This is a rare disease of unknown etiology, which may be dependent on a constitutional abnormality of bone development, and is sometimes familial. It begins in early life, although it may not be recognized until the third decade. It is characterized by a peculiar type of irregular thickening of the bones, involving especially the shafts of the long bones. The new bone encroaches on and eventually may largely obliterate the marrow, which shows in addition extensive fibrosis. In spite of the thickening, the bones are fragile, and gross deformities and pathological fractures are common. In the later stages a severe anaemia develops (as low as R.B.C. o.82, Hb. 10%, Vaughn), characterized by a marked erythroblastosis. The spleen and liver are much enlarged as a result of myeloid hyperplasia.

Myelosclerosis is a term applied by Mozer (1927) to a somewhat similar condition developing in adults, in which the bones become abnormally dense but not widened, and the cellular marrow is replaced by fibrous tissue. Eventually a severe anaemia develops, with erythroblastosis and splenomagaly.

# C. Anaemias Associated with Accelerated Blood Destruction (Haemolytic Anaemias)

Although accelerated blood destruction is a prominent feature of these diseases, in many of them inadequate blood formation is also important in the production of the anaemia. In some of them, as in pernicious anaemia, sickle cell anaemia, and probably haemolytic jaundice, the primary disturbance appears to be the formation of red cells which are inherently defective, and thus susceptible to the normal processes for removal of damaged cells. There is no positive evidence that anaemia is produced by removal and destruction of normal cells, because of a pernicious overactivity of the reticuloendothelial tissues.

In nearly all cases red cells which are defective or which have been damaged are removed from the circulation before haemolysis occurs. The mechanism of this process has been described in the previous chapter. If the rate of red cell destruction is accelerated (or if the liver is injured), the liver may fail to remove the bilirubin from the serum as fast as it is formed. As a result bilirubin accumulates in the plasma and gives the latter a yellow color. The icterus index rises, and the serum gives a positive (indirect)

van den Bergh reaction. The skin and sclerae become more or less jaundiced. In some cases this may be deep, but usually it is relatively slight, and the color is a pale lemon-yellow rather than the orange tint of obstructive jaundice. The difference, however, is purely a quantitative one. The color in both cases is due to bilirubin. The urine becomes dark colored, and contains increased amounts of urobilin and urobilinogen, but no bilirubin (or only traces). The sediment may show renal epithelial cells containing haemosiderin granules. The urobilin in the faeces is increased. At autopsy the amount of iron in the liver and spleen is increased. These phenomena are marked only during periods of rapid blood destruction. If the latter is relatively slow, they will be slight, and perhaps limited to a small increase in the bilirubin in the serum.

The rapid destruction of red blood cells in the body, regardless of the disease in which it occurs, gives rise to a characteristic clinical syndrome. There is fever, sometimes a chill, weakness and prostration, pain in the back, and crises of acute colicky abdominal pain, with nausea, vomiting, and jaundice. It may simulate various acute abdominal conditions, and has led to unnecessary operations.

In rare instances in which a large number of red cells are rapidly destroyed, the cells may be haemolyzed in the circulation and the haemoglobin liberated into the plasma (haemoglobinaemia). If the amount of haemoglobin so liberated is large (when about ½00 or more of the red cells are abruptly destroyed), haemoglobinuria occurs.

This is met with: (1) After transfusions of incompatible blood. (2) In paroxysmal haemoglobinuria. (3) In black-water fever. (4) Rarely in severe infections and intoxications of the types enumerated below (as in gas bacillus gangrene, and poisoning with arseniuretted hydrogen), and in favism. (5) In march haemoglobinuria. (6) In paroxysmal nocturnal haemoglobinuria.

- A haemolytic anaemia, usually without haemoglobinaemia and haemoglobinuria, is met with chiefly in the following conditions:
- 1. Some acute infections, as gas bacillus gangrene, sepsis, especially puerperal sepsis and other streptococcal infections, typhoid fever, malaria, and Oroya fever. Rarely a profound anaemia may develop within a few hours.
  - 2. Acute (febrile) haemolytic anaemia of Lederer.
- 3. Some cases of acute poisoning with certain drugs: phenol, benzol, and their derivatives, nitrobenzene, trinitrotoluene, phenylhydrazine, aniline, acetanilid, sulfanilamide; saponin; potassium chlorate; lead and other heavy metals; arseniuretted hydrogen; snake venom; etc.
  - 4. Extensive burns.
- 5. Pernicious anaemia and related macrocytic anaemias during acute exacerbations of the disease.
  - 6. Haemolytic jaundice.
  - 7. Sickle cell anaemia.
  - 8. Icterus gravis neonatorum.
  - 9. Cooley's erythroblastic anaemia.

The morphological changes in the red cells in anaemias of this type are not distinctive, except in the specific diseases pernicious anaemia, haemolytic jaundice, and sickle cell anaemia. Anisocytosis and anisochromia are usually moderate and are typically less than in chronic post-haemorrhagic anaemia of the same degree. The volume index (mean corpuscular volume) and color index may be somewhat reduced, but more often they are within normal limits, and occasionally they are increased. These anaemias are not hypochromic, probably because the iron from the cells which have been destroyed is retained in the body and is readily available for the production of new haemoglobin. Evidences of active red cell formation, reticulocytes, polychromatophilic or stippled red cells, even normoblasts, are usually present and may be numerous, as in lead poisoning. In acute cases there is usually a leukocytosis and an increase in platelets. In protracted chronic cases the blood may finally assume the features of an aplastic type of anaemia.

Paroxysmal haemoglobinuria is characterized clinically by recurring brief paroxysms of chills, fever, pain in the back, prostration, cramps, vomiting or diarrhoea, numbness in the extremities, and haemoglobinuria. The individual attacks are usually precipitated by exposure to cold, or experimentally by holding the arm in ice water. They are followed by jaundice and (haemolytic) anaemia which may be severe. (R.B.C. 1.0.)

The haemolysis is brought about by an haemolysin of the usual complex type. The stable constituent ("amboceptor") is peculiar in that it will combine with cells only at a low temperature. Once combined, it renders the cells susceptible to the lytic action of complement when warmed to body temperature (the *Donath-Landsteiner phenomenon*).

No.	Serum, 0.5 cc.	Cell suspension, 0.2 cc.	Complement, cc.	Salt sol., cc.	Positive result
ı	Patient's	Patient's	0.2	1 0	Haemolysis
2	Control	Control	0 2	0 1	0
3	Patient's	Control	0.2	O. I	Haemolysis
4	Control	Patient's	0.2	O. I	0
5		Patient's	0.2	0.6	0
6		Control	0.2	0 6	0

The hacmolysin can usually be demonstrated in the following simple manner. Put about 1 cc. of fresh (warm) blood in each of two test tubes and keep at body temperature until the serum has separated. Keep one tube in the water bath at 37°C. as a control and put the other in ice water for 5 to 7 minutes. Then put this in the water bath for an hour. If the serum in the control tube remains colorless, the presence of the haemolysin is indicated by more or less tingeing of the serum in the second tube. If the result is doubtful, add to each tube a little additional complement and return to the water bath. (Use 1 to 10 dilution of fresh guinea pig serum, or fresh normal human serum of the

same blood group; this is usually necessary if the blood has been chilled for more than 10 minutes.)

For more precise demonstration withdraw about 10 cc. of blood from the patient in a warm syringe, allow about 8 cc. to clot (for serum) and oxalate the rest. The blood, solutions and apparatus must be kept at body temperature throughout all manipulations. Wash the cells and prepare a 5% suspension. Secure 10 cc. of normal human blood of the same blood group and treat in the same way. Prepare a 1 to 10 dilution of fresh guinea pig serum as complement. Set up tubes as shown on page 361.

Put all tubes in ice water for 10 minutes and then in the water bath at 37°C. for half an hour. A positive reaction is indicated by haemolysis in tubes (1) and (3) and by absence of haemolysis in all the other tubes.

The condition is regarded as a rare late manifestation of syphilis, since a large proportion of patients give a positive Wassermann reaction, and it can sometimes be cured by adequate treatment of the syphilis. The haemolysin has been observed in some cases of late syphilis, who show no clinical symptoms of paroxysmal haemoglobinuria, but not in other conditions.

The disease must be differentiated from black water fever and other rare forms of haemoglobinuria. *March haemoglobinuria* may follow unusual muscular exertion, such as long marches or standing for hours in a lordotic posture.

Paroxysmal paralytic "haemoglobinuria" (similar to a relatively common disease of horses) has been reported in a few human cases. There are recurring attacks of extreme muscular weakness, followed by "haemoglobinuria," and later by more or less marked muscular atrophy but little or no anaemia. The pigment excreted is myoglobin. The muscles in fatal cases show marked degenerative changes and loss of pigment ("fish flesh"). A somewhat similar acute degeneration of striated muscle with myoglobinuria has been observed in Königsberg ("Haffkrankheit") in individuals (and in cats) who had eaten fish which had ingested poisonous resinous acids, the waste products of cellulose factories.

Paroxysmal nocturnal haemoglobinuria is a chronic relapsing disease of unknown etiology characterized by an insidious onset with weakness, anaemia, jaundice and later recurring attacks of haemoglobinuria without obvious exciting cause, which usually occur at night and lead to severe anaemia. The blood shows a leukopenia, many reticulocytes, and normal fragility. Splenectomy has not been beneficial. (Reviewed by Hamburger and Bernstein, 1936.)

In none of these conditions is there any relation to syphilis or to exposure to cold, and the Donath-Landsteiner phenomenon is absent.

Lederer's acute haemolytic anaemia is an acute febrile disease of unknown cause, presumably an acute infection, affecting chiefly children and young adults. It is characterized clinically by the rapid development of a severe haemolytic anaemia (R.B.C. 1.0) with great prostration, jaundice, abdominal pain, vomiting and diarrhoea, often purpura and haemorrhages, occasionally anuria and uraemia. There is usually a leukocytosis, and often many myelocytes and some myeloblasts appear in the blood. It is often fatal if not properly treated, but can usually be cured by prompt and repeated transfusion. It has been mistaken for acute pernicious anaemia and acute leukaemia.

Lead poisoning frequently causes an anaemia which is usually moderate in degree (R.B.C. 3.0 to 4.0), but may become severe. In severe cases it may cause an acute haemolytic anaemia. The most characteristic feature

of the blood is the appearance of many reticulocytes and stippled cells. These cells may appear within a few days after exposure to lead, in the absence of appreciable anaemia. Although stippled cells may appear in any anaemia in which active red cell regeneration is taking place, and although they may be sparse in some cases of lead poisoning, their early appearance and their presence in large numbers is highly characteristic.

Actual counts of stippled cells have been utilized to detect and measure the degree of absorption of lead in industrial workers. In Germany a count of from 100 to 300 per million red cells has been regarded as an indication for enforced change of occupation. Belknap (1935) has found that men with counts of 500 to 1000 per million might continue at work for years without clinical symptoms of lead poisoning, but that an abrupt increase above these figures was usually followed by acute symptoms. Counts of 40,000 per million, and more have been observed. The number may be estimated by counting the number of stippled cells in 50 oil immersion fields in an ordinary thin film, and multiplying this figure by 100 (the average number of red cells per field is about 200, and this should be roughly checked by those not experienced in such counts). McCord's basophilic aggregation test (p. 308) should be used if the cells are sparse.

An increase in *reticulocytes*, although less specific, is an earlier and more sensitive sign of lead absorption than the appearance of stippled cells. There is a rough parallelism

between the counts of the two types of cells. Jones (1935) found the number of reticulocytes trebled with 100 stippled cells per million, and 5 times the normal with 1000 stippled cells per million. Reticulocyte counts of 16% and more have been observed in acute poisoning. Jones found appreciable numbers of normoblasts in about 5% of the chronic cases.

The resistance of the red cells to hypotonic salt solution is increased, but the cells appear to be abnormally fragile and susceptible to mechanical injury (Aub).

Acetanilid and related drugs in overdose cause a transformation of haemoglobin to methacemoglobin. This imparts to the mucous membranes a characteristic, dusky, cyanotic tinge. It can be recognized by spectroscopic examination, but must be differentiated carefully from sulphaemoglobin which gives a closely similar

Fig. 75.—Sickle cell anaemia. Severe case showing an unusual number of sickled cells in a stained film. Three normoblasts. Upper left: Macrophage containing a red corpuscle.

spectrum. As a rule, after removal of the poison there is a reversion to normal haemoglobin, without much injury to the red cells. More rarely it causes a severe acute haemolytic anaemia, associated with a leukocytosis and occasionally an erythroblastosis.

Sickle cell anaemia is an hereditary constitutional anomaly practically limited to negroes, transmitted by either sex as a dominant Mendelian

characteristic, and characterized by the tendency of the red cells (in sealed fresh preparations) to assume characteristic bizarre shapes. The cytoplasm of the cells at two or more points becomes drawn out into elongated spine-like projections, so that the cells become crescentic, or more often oat-shaped or irregularly stellate. Hahn and Gillespie found that the cells would resume the normal shape if oxygen was supplied to the preparation, and would again "sickle" if it was withdrawn. These distortions are not seen in ordinary fixed films, except to a slight extent in a few cells in the severest cases.

A majority of the individuals who show this trait are symptomless. In a few cases recurring attacks of acute haemolytic anaemia occur, with partial recovery in the intervals. In addition to the usual symptoms due to acute haemolysis, older patients often complain of deep seated pain in the bones and joints, often associated with osteoporosis in roentgenograms; and chronic punched-out ulcers over the lower legs. The skull may show changes similar to those in Cooley's anaemia. The spleen is often enlarged in young children, later it becomes small and fibrotic. Symptoms appear in childhood (if at all), and if severe, the prognosis is unfavorable. Retardation of development, both mental and physical, is common.

The degree of anaemia is variable, but it may be profound (R.B.C. 1.0 or less). The color index and volume index vary, but usually are about 1.0. There are many reticulocytes and polychromatophilic cells, and often many normoblasts. The fragility of the red cells is normal. There is usually a leukocytosis and an increase in platelets. In severe cases monocytes containing phagocyted red cells can often be found.

Elliptical red cells, which occur as a rare familial trait in Caucasians, must be sharply differentiated. The cells do not "sickle," and the condition does not cause anaemia or impair the health.

**Haemolytic Jaundice.**—Two types have been described: (1) a congenital familial form, and (2) an acquired form, occurring later in life, and without a familial history.

Familial haemolytic jaundice depends upon a constitutional anomaly which is transmitted by either sex as a dominant Mendelian characteristic. The disease becomes manifest during the first or second decade, and is characterized by recurring attacks of haemolytic anaemia, with the usual symptoms of acute haemolysis and outspoken jaundice. The serum bilirubin may be increased to from 10 to 50 times the normal. In the intervals there is partial recovery, but some anaemia and jaundice persist. The acute attacks may be precipitated by an acute infection or other associated disease, but often no exciting factor can be found. Gall stones develop in about half the cases, and gall stone colic and obstructive jaundice may mask the underlying disease. The spleen is regularly enlarged. The bone marrow shows a marked hyperplasia which is usually normo-

blastic. Extra medullary areas of hyperplasia may occur. The disease causes marked disability, but is rarely directly fatal.

The degree of anaemia is usually slight or moderate, but may be marked. The average red cell count is from 3.0 to 3.5. The color index and volume index (and mean corpuscular volume) are normal or slightly increased. The distinctive features are: (1) The diameter of the red cells is diminished, but the thickness is increased, so that they are more globular than normal. Krumbhaar has called the disease "spherocytic icterus." (2) The resistance to hypotonic salt solution is diminished. Haemolysis usually begins in concentrations from 0.5% to 0.6%, rarely even 0.8% (instead of 0.44%), and may be complete at 0.4% to 0.48% (instead of 0.34%). Not infrequently, however, the divergence from normal is relatively slight. (3) Reticulocytes are much increased, often to 10% or 20%, rarely to 50% and more. Other evidences of regeneration are present, including frequently a few normoblasts.

Splenectumy stops the rapid cell destruction and usually effects a permanent clinical cure, although the abnormal shape and diminished resistence of the cells persist in some degree. Relapses have occurred, and in some cases have been associated with hyperplasia of accessory spleens.

Not infrequently examination of the relatives of a patient reveals *latent cases*, with a slightly diminished resistance of the red cells to hypotonic salt solution as the only manifestation of the anomaly.

Acquired haemolytic jaundice differs fundamentally from the familial type only in the late onset and apparent lack of hereditary factors. The condition is usually regarded as secondary to some infection or other organic disease (which is not always demonstrable). Clinically the disease is usually more severe than the preceding type. The haemolytic crises are more acute, the anaemia more profound (R.B.C. average 2.0, minimum 0.5), and a fatal outcome common. Splenectomy is less regularly effectual. The globular shape and diminished resistance of the red cells is often less clear cut than in the familial type. These features constitute the only decisive characteristic by which this condition can be differentiated from ordinary "secondary" haemolytic anaemias. Some question its existence as a distinct disease.

Congenital erythroblastic anaemia (Erythroblastosis foetalis).—This is a congenital, familial (but not directly hereditary) disease of unknown etiology. It appears in three distinct clinical types which probably represent different stages, or different degrees of severity, of the same process (Diamond, Blackfan and Batty, 1933). Successive children in the same family are often affected, and may show different types of the disease. All have the following characteristics in common: (1) A severe hypochromic type of anaemia (R.B.C. often 1.0 or less). (2) Extraordinary numbers of circulating erythroblasts (up to 50,000 per cmm.) of every stage of maturity. (3) Extreme hyperplasia of erythropoietic tissue, both intramedullary and extramedullary, in the spleen, liver, and many other organs. (4) Great enlargement of the spleen and liver. (5) A leukocytosis with many immature cells. (6) Often a bright golden-yellow vernix caseosa and amniotic fluid.

- a. Hydrops foetalis.—In this type the infant is still-born or dies within a few hours. There is marked generalized oedema of the placenta and foetal tissues, cardiac dilatation, dyspnoea and cyanosis.
- b. Icterus gravis neonatorum.—There is jaundice, which may be present at birth, or may appear during the first 12 to 48 hours. It increases rapidly to a deep orange-brown color. Petechiae may occur. The disease, if untreated, is often fatal within the first week, but it can usually be cured by repeated small transfusions, or (Hampson, 1929) by intramuscular injections of normal human serum. Recovery, if it occurs, is complete and permanent. The tissues are deeply icteric, including in some cases the basal ganglia ("Kernicterus"), and there are extensive deposits of iron pigment.
- c. Congenital anaemia of the new born is practically identical with the preceding type except for the absence of jaundice. It is distinguishable from the simple hypochromic anaemias chiefly by the erythroblastosis and splenomegaly.

The erythroblastic anaemia of Cooley is a congenital and often familial constitutional anomaly largely limited to children of eastern Mediterranean races. It is characterized by (1) a hypochromic type of anaemia which becomes severe (R.B.C. 1.7, Hb. 10%); (2) mild jaundice, with hyperbilirubinaemia; (3) a leukocytosis; (4) a marked erythroblastosis (often 100 or more per 100 W.B.C.); (5) a marked generalized hyperplasia of erythropoietic tissue with enlargement of the liver and spleen; and (6) peculiar, characteristic changes in the bones, especially the skull. The cortex is thinned, and the medullary portion becomes greatly widened and porous, so that in roentgenograms the trabeculae stand out like fine sharp spines. The thickening of the bone is so great that it gives the patients a characteristic mongoloid physiognomy with high, bulging forehead and prominent malar eminences.

The anaemia is not noted at birth, but becomes evident within the first year or two. The disease runs a chronic, slowly progressive course and is usually fatal within the first ten years. Splenectomy has proved useless, and is followed by a great increase in the erythroblastosis (up to 1500 nucleated red cells per 100 W.B.C.).

### MISCELLANEOUS CONDITIONS

Banti's disease is a disease of unknown cause, occurring chiefly in young adults, and characterized by the gradual development of a hypochromic anaemia, associated with splenomegaly, progressive weakness and emaciation, a tendency to gastric haemorrhages, and a terminal atrophic cirrhosis of the liver, with ascites and jaundice. The anaemia is usually moderate (R.B.C. 3.0, Hb. 50%), but evidences of red cell regeneration are scanty. At times there are evidences of increased red cell destruction. The fragility of the red cells is normal. There is usually a neutrophilic leukopenia and a moderate reduction in platelets. Splenectomy in the early stages of the disease is usually curative, or at least temporarily beneficial. The advisability of this procedure has been questioned in cases with normal platelets because of the frequency of postoperative thrombosis in this group.

The cases commonly included under this heading constitute a heterogeneous group, and many investigators deny the existence of Banti's disease as a definite entity

Similar pathological changes may follow occlusion of the splenic or portal veins from various causes. The condition should be regarded as a clinical syndrome rather than as a distinct disease.

Anaemias in young children, regardless of their cause, often differ from those in adults in the type of cellular response. Immature red cells are more numerous, particularly normoblasts, macroblasts, and even megaloblasts. There is more regularly a leukocytosis (or lymphocytosis), which may be marked and accompanied by many immature leukocytes. There is often enlargement of the spleen, liver, and lymph glands, due to erythroblastic hyperplasia in these organs. The anaemia pseudoleukacmica infantum of Von Jacksch represents in marked degree such an infantile response to anaemia resulting from a variety of infectious diseases and metabolic or nutritional disturbances.

### Робусутнаеміл

By a polycythaemia, or more precisely an *crythrocytosis*, is meant an increase above the normal in the number of red cells per cmm. of blood.

In a relative polycythaemia there is no increase in the total number of red cells in the body. It is seen chiefly as a transient phenomenon associated with dehydration, and is a rough measure of its severity. A local erythrocytosis may occur as a result of local stasis, whether due to chilling and acrocyanosis, or to the use of a tourniquet.

In an absolute polycythaemia there is an increase in the blood volume as well as in the red cell count. There is a pathological increase in the total number of red cells in the body. This occurs (1) in the specific disease erythraemia (polycythaemia vera), and (2) as a compensatory response to anoxaemia in the following conditions (secondary polycythaemias):

- 1. Normal individuals at high altitudes.—Within an hour or two after a sudden ascent (as in aviation) there may be a rise of 500.000 red cells per cmm., due to an outpouring of cells from the spleen, which disappears promptly after descent to normal levels. With a sojourn at high altitudes, in some individuals there is an increased production of red cells, with a transient rise in reticulocytes, which is stimulated by the low O tension. The red cell count may reach 8 million or more. In rare instances typical crythraemia has ensued.
- 2. In chronic myocardial insufficiency with cyanosis, as in some cases of mitral stenosis and congenital pulmonary stenosis.
- 3. In emphysema, in diffuse pulmonary fibrosis from any cause, and in sclerosis of the pulmonary arteries (.1yerza's disease).
- 4. After mild chronic poisoning with a variety of blood poisons, such as lead, carbon monoxide, etc.

Erythraemia, or polycythaemia vera (rubra), is a chronic disease of unknown etiology, characterized by an erythrocytosis with an increase in total blood volume, by a peculiar intense flushing of the skin and mucous membranes, by enlargement of the spleen, and by a normoblastic

SPLENOMEGALY IN DISEASES OF THE BLOOD, DIFFERENTIAL DIAGNOSIS

	Time of onset	Duration	Spleno- megaly	Pathology of Spleen	Splenec- tomy beneficial	Haemor- rhages	Anaemia	Leukocyte count
Pernicious anaemiaAdult late	Adult late	Many	Slight, occa-	Fibrosis	No	Rare	Severe	Low
Idiopathic hypochromic anaemia.	. 70-20	years Many	Slight in 25-	~-	°N °N	Rare	Moderate to	Normal or
Familial haemolytic jaundice	Congenital or	years Life	30 % ++	Congestion pig-	Usually	Rare	Slight to se-	low Variable
Acquired haemolytic jaundice	childhood Adult	Many	++	Congestion pig-	Often	Rare	Severe	Variable
Banti's disease	years Childhood; young Several	years Several	+	mentation Hyperplasia	Often	Gastric com-	Slight to se-	Low
von Jaksch anaemia	adult Infancy	years Several	+++	fibrosis Hyperplasia	In some	mon Rare	vere Often severe	Increased
Idiopathic thrombopenic purpura. Variable often in	Variable often in	months Many	Rare	fibrosis None character-	cases Often cur-	++++	Severe after	Variable of-
Anaphylactoid nurnura	childhood	years Brief at-	+ in some	istic None character-	ative No	Trivial	bleeding No	ten reduced Increased
4		tacks	cases	istic				
Polycythaemia	Adult late	Several	+	Congestion hy-	Probably homeful	Occasional	No	Increased
Chronic myeloid leukaemia	Adult	years 2–5 years	++++	perpiasia Myelocytic in- filtration		Occasional	Becomes se-	Very high
Chronic lymphatic leukaemia Adult	Adult	2-10	+++	Lymphocytic in- No	°N	Rare	Becomes se-	Usually very
Acute leukaemia (myelogenous,		A few		Primitive cell		Common	2	Low, normal
lymphatic, monocytic)			+ ]	infiltration	Harmful	+++		or high
Hodgkin's disease	Any age	1-5 years	+	recunar nyper- plasia, fibrosis	ONT	No No	become se-	varianse
Lymphosarcoma	Any age	1-3 years	ı	Hyperplasia of	No	No	Slight to	Usually
Gaucher's disease Infancy	Infancy	Life	+++	Hyperplasia of	No	Occasional	moderate Slight	Normal or
				R.E. (foam)				low
				cells				

hyperplasia of the erythropoietic tissues. It affects chiefly adults over 50 years of age. It is characterized clinically by an insidious onset and a protracted chronic course, with weakness, headaches, vertigo, tinnitus, paraesthesias, nervous irritability and mild mental disturbances. The skin, especially of the face and extremities, acquires an intense, mottled, brick-red color, the mucous membranes a deep purplish red or plum color, due to marked dilatation of the superficial capillaries together with slowing of the local circulation. Haemorrhages and thromboses are common. There is often hypertension, and resulting myocardial insufficiency. Occasionally cirrhosis of the liver develops, or arteriolosclerosis of the kidney and renal insufficiency. The basal metabolic rate is increased in about half the cases.

The blood at some stage of the disease usually shows a red cell count of 8 million or more, rarely even 12 to 15 million. However, the count is not invariably or constantly so high. In undoubted cases it may be between 5 and 6 million. The blood volume is always increased and may be double the normal, a decisive point in doubtful cases. The haemoglobin is increased to a relatively less degree; usually 19 to 24 Gm., 130% to 160%. The viscosity is from 2 to 5 times the normal. The individual red cells are somewhat small and pale, they show slight anisocytosis, and usually a moderate increase in reticulocytes and other immature forms. Platelets are increased. There is a moderate leukocytosis, which may be marked and associated with a slight myelocytosis. Rarely in the terminal stages the blood may show an aplastic anaemia, or the presence of many myelocytes may suggest a myeloid leukaemia.

Symptomatic relief with restoration of a normal blood count may follow radiation of the long bones, or more certainly the *cautious* administration of phenylhydrazin.

The resemblance of the symptoms and changes in the blood to those of mountain sickness has suggested that there is an anoxaemia of the tissues in erythraemia. However, no mechanism for the production of such an anoxaemia has yet been demonstrated, except that thickening of the walls of the capillaries has been described. Some regard the process as a malignant hyperplasia, analogous to leukaemia.

### DISEASES INVOLVING PRIMARILY THE LEUKOCYTES

### The Leukaemias

Leukaemia is a disease characterized by an abnormal proliferation of the leukopoietic tissue, and by the appearance in the circulating blood of immature leukocytes which are not present in normal blood. There is usually a marked increase in the number of circulating leukocytes. There are three distinct types of leukaemia, termed myelogenous, lymphatic, or monocytic, depending upon which of the leukopoietic tissues is involved. Clinically, acute and chronic forms of each type may be differentiated, depending upon the mode of onset and the duration of the disease. Cases living less than 4 months are arbitrarily classed as acute, but every gradation occurs. The cause of the disease is unknown. Many regard it as a special type of malignant growth. All forms of leukaemia are rare.

Chronic myelogenous leukaemia, the most common type, is a disease of adults, characterized clinically by an insidious onset, with symptoms referable to the enlarged spleen, and progressive weakness, emaciation, and anaemia. Symptoms of myocardial insufficiency and digestive dis-

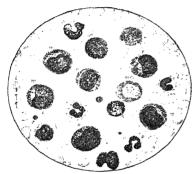


FIG. 76.—Chronic myelogenous leukaemia. m, Myelocyte; p, polymorphonuclear; b, mast cell; n, normoblast. (Cabol.)

turbances frequently occur, and occasionally haemorrhages, pruritus, priapism, and pain and tenderness in the long bones. The spleen is huge, often reaching to the right anterior superior spine. The liver is usually much enlarged, the lymph glands rarely.

The *blood* shows a total leukocyte count which is usually between 100,000 and 500,000 per cmm., but rarely it may reach 1.0 or 1.5 million. Occasionally it is within the range of an ordinary leukocytosis. As a rule the bulk of the leukocytes are polymorphonuclear neutrophiles and neutrophilic metamyelocytes (30% to 65%)

Neutrophilic myelocytes are always present (5% to 70%, usually 20% to 50%. Eosinophiles and basophiles are increased, and occasionally either type of cell may be markedly increased (up to 40%). The corresponding types of myelocytes are present, and there may be an occasional primitive myelocyte or myeloblast. Pathological leukocytes of bizarre appearance are often found. The lymphocytes and monocytes are relatively reduced.

The platelets are much increased.

The red cell count is reduced, and is usually from 1.0 to 3.0 million. The mean corpuscular volume and haemoglobin content are about normal. Immature red cells are increased, and normoblasts are constantly present, often in fairly large number; rarely, a few megaloblasts.

Remissions may occur, either spontaneously, or in association with an acute infection, or after radiation or other effective therapy. During a remission, or in the incipient stages of the disease, the total leukocyte count may be normal (aleukaemic myelosis), and the diagnosis may be missed without a careful differential count. This almost always reveals myelocytes (5% to 30%) and normoblasts.

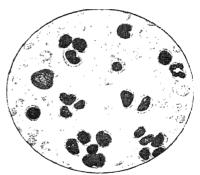
The disease often terminates with an acute exacerbation, during which the blood takes on the characteristics of an acute leukaemia (described below). This is ushered in by fever, an abrupt aggravation of the clinical symptoms, a rapid fall in red cell count and haemoglobin, often thrombocytopenia and purpura, and the appearance of many primitive myelocytes and myeloblasts. The total leukocyte count may rise or fall.

Difficulties in diagnosis occur chiefly in the hyperleukocytoses due to infection, haemorrhage, etc., and in focal lesions of the bone marrow when these are associated with a stimulation myelocytosis.

Chronic lymphatic leukaemia is also a disease of adults, which resembles the preceding type in its symptoms and clinical course except that there is a generalized enlargement of the lymph glands, tonsils, and other

aggregations of lymphoid tissue. Local infiltrations in the skin and membranes are common. mucous Splenomegalv is less marked.

The blood shows an increase in the total leukocyte count which may range from 10,000 to 2.5 million, but in most cases is from 100,000 to 200,000, somewhat lower than chronic myelogenous leukaemia. Counts under 50,000 are not uncommon. From 90 to 90% of the cells are lymphocytes, practically all small cells with nuclei of mature type containing dense chromatin masses. The cyto- megaloblast; c, eosinophile. plasm is often scanty, and azure gran-

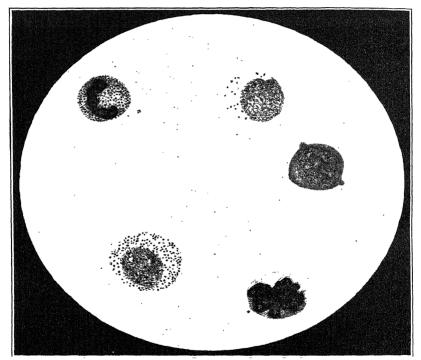


77.-Chronic lymphatic leup, Polymorphonuclear; m, one lymphocytes in this field.

ules are sparse or absent. There are usually a few lymphoblasts, some of which may contain lobulated or indented nuclei (Rieder cells). cytes may be absolutely, as well as relatively, reduced. The platelets become diminished. In the later stages an aplastic type of anaemia develops. Normoblasts are rare.

Cases occur, anatomically and clinically identical, except that the total leukocyte count is normal. They usually show a relative lymphocytosis. The blood may become frankly leukaemic at any time, or death may ensue before this occurs (aleukaemic lymphadenosis, "aleukaemic" lymphatic leukaemia).

Diagnostic difficulties arise chiefly in infections with a high lymphocytosis, such as infectious mononucleosis and whooping cough; and in diseases associated with generalized enlargement of the lymph glands and spleen (Hodgkin's disease, lymphosarcoma). Cases of lymphatic leukaemia with a relatively low total leukocyte count are difficult to recognize by an examination of the blood alone, because the differences between mature and immature lymphocytes are less conspicuous than in the case of the granulo-



Fro. 78.—Acute myelogenous leukaemia. (W.B.C. 13,000.) Actual field. Right: myeloblast. Upper right: promyelocyte. Lower left: myelocyte. Lower right: myeloblast in mitosis.

cytes and because lymphoblasts appear in the blood more frequently than myelocytes in conditions other than leukaemia.

Acute Leukaemia.—This may be myeloid, lymphoid, or monocytic in type. Clinically the three types are practically indistinguishable. The disease occurs chiefly in children and young adults. In about half the cases of acute leukaemia the onset is preceded by an acute infection, or there is a history of repeated chronic infections. In some cases the sequence is so direct that one can scarcely escape the conclusion that the

leukaemia represents a perverted (irreversible) response to the stimulus of the infection. Clinically these cases can not always be differentiated sharply from patients with a (reversible) "leukaemoid reaction" to infection. Diagnosis may be possible only by biopsy of the marrow, or by observing the ultimate outcome. However, some

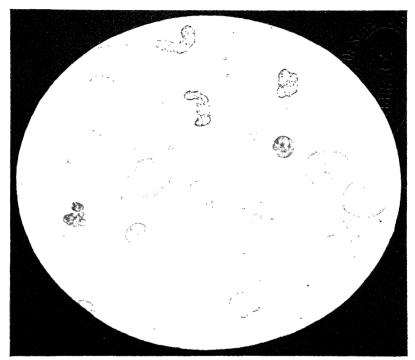


FIG. 79.—Acute leukopenic myelogenous leukaemia. (W.B.C. 3,000.) Film from buffy coat (see text). Actual field. Five myeloblasts, several showing nucleoli. Lower left: promyelocyte. Three non-segmented neutrophiles, practically destitute of granules (toxic-degenerative changes). Lower right: two megakaryocyte nuclei with shreds of platelet material attached.

individual susceptibility or defect of the marrow must be assumed. The infection can at most be regarded as only an exciting cause of the leukaemia.

In some cases there is a preliminary period of vague ill health, weakness, and pallor. Usually the onset is fairly abrupt, and may be fulminant, with fever, weakness, prostration, and often sore throat, followed quickly by an ulcerative stomatitis and gingivitis, and an haemorrhagic diathesis with purpura and oozing from the gums and other mucous membranes, and a rapidly developing anaemia. Clinically it resembles an

acute septic infection. There is progressive enlargement of the spleen. Death usually occurs within 2 to 4 months, and may occur within two weeks. Remissions, however, may occur.

The leukocyte count in the early stages may be normal or moderately increased. There may be a well marked leukopenia, ('aleukaemic leukaemia,' or better, leukopenic leukaemia). As a rule there is a progressive increase in the count, which often exceeds

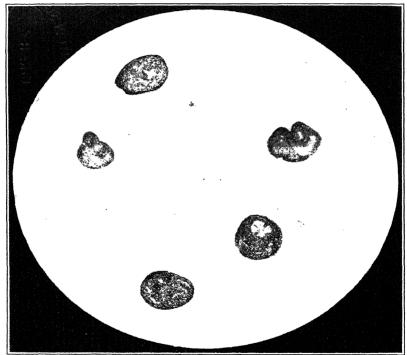


Fig. 80.—Acute lymphatic leukaemia. Actual field. Five lymphoblasts, showing nucleoli. Upper right: Rieder cell.

100,000 within a few weeks. The characteristic feature is the appearance in the blood of increasing numbers of primitive leukocytes. The platelets are much reduced.

In the leukopenic cases the study of the cells is greatly facilitated by drawing blood from a vein into one third its volume of 1.5% sodium citrate solution, centrifugalizing and making films from the buffy coat (see Fig. 70).

In acute myelogenous leukaemia, the commonest type, the primitive cells are promyelocytes or myeloblasts. There is not a gradual transformation or transition from normal leukocytes, through myelocytes of varying stages of immaturity, to myeloblasts.

In the early stages of the disease the primitive cells appear, at first in small numbers, as intruders in a leukocyte population which is essentially normal. As the myeloblasts increase in number the mature leukocytes are rapidly crowded out, and may entirely disappear. The exact type of primitive cell varies considerably in different patients, but is usually quite uniform and constant in the same case. Careful search usually reveals a few cells which are somewhat more mature (myelocytes), a finding which may be of great assistance in identifying the type cell. Normoblasts are regularly present, occasionally megaloblasts.

Fig. 81.—Acute monocytic leukaemia. Actual field. Eight of the cells are monocytes. They show a grey-blue cytoplasm with fine reddish lilac granulations, and nuclei of finely reticular structure with various types of infoldings and convolutions. Upper left: a hypersegmented polymorphonuclear neutrophile. Below it, a monoblast with more basophilic non-granular cytoplasm and a more finely reticulated nucleus with several small nucleoli. Below this, a monocyte showing several pseudopods containing only clear ectosarc. Right center, a plasma cell. On extreme right, a smudged nucleus, Wilson's stain. X800. (From the Johns Hopkins Hospital Bulletin.)

Acute lymphatic leukaemia is very rare except in children. The predominant cells are lymphoblasts. Cases with marked leukopenia occur. Progressive enlargement of the lymph glands usually occurs, and in some cases they form large tumor-like masses. Rarely the lymphoid hyperplasia may be limited largely to the marrow and the deeper glands.

Monocytic leukaemia usually runs an *acute* course. It occurs in individuals of any age. Clinically this type is characterized by marked hyperplasia (and ulceration) of the gums, which may become so great as to engulf the teeth; by the absence of lymph-glandular enlargement; and often by excessive bleeding, and a rapidly fatal course. The

leukocyte count ranges usually from 20,000 to 400,000. The predominant cells (20% to 90%) are monocytes and their precursors. Their differentiation from early myelocytes is sometimes difficult. (See Clough, 1931, for discussion and illustrations of cells.) Motility and phagocytic activity in fresh preparations is decisive evidence that they are monocytes. Myelocytes and myeloblasts are usually present in small numbers, and in some cases they are numerous, and give the appearance of a "mixed leukaemia." This has led Naegeli and others to question the existence of monocytic leukaemia as a distinct disease. With this possible exception, there is no evidence of the occurrence of true "mixed leukaemia," with simultaneous involvement of two or more of the leukopoietic tissues, nor of transformations from one type of leukaemia to another.

There is an extraordinarily wide-spread, diffuse hyperplasia of the reticulum cells in the tissues.

Cases with a normal leukocyte count or a leukopenia occur, but they usually show a relative monocytosis (aleukaemic reticulo-endotheliosis).

# Special Types of Leukaemia

Chloroma is a rare type of leukaemia in which localized, invasive, tumor-like growths of leukopoietic tissue occur. The growths are most often connected with the bones of the skull, especially the orbit, and may cause bony deformities and often protrusion of the eye balls. They often cause intense pain or paralyses from pressure on neighboring structures. The tumor masses on section (in gross) usually have a green color. The changes in the blood and the clinical course are otherwise identical with acute or subacute myelogenous leukaemia. All transitions to the ordinary type of leukaemia occur.

Eosinophilic leukaemia and mast cell leukaemia are terms sometimes applied to cases of chronic myeloid leukaemia which show an unusually large percentage of one of these types of cells. Aside from these cases, which are only minor variants of ordinary chronic myelogenous leukaemia, there are records of a few cases in which enormous numbers of mature eosinophiles were present, without other significant abnormality in the leukocytes. These cases ran the course of a subacute (rarely chronic) leukaemia, and at autopsy showed leukaemic infiltration of the tissues with eosinophiles (Hay and Evans, 1920).

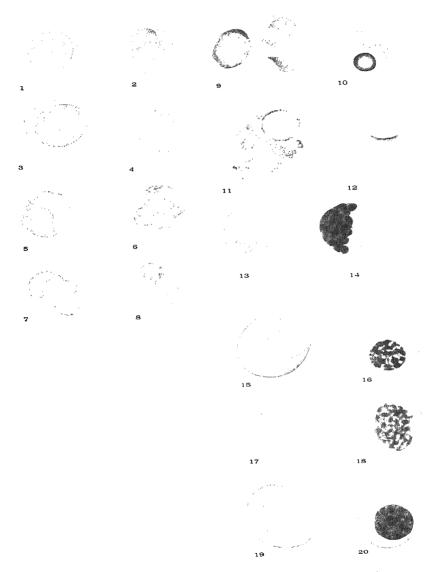
Plasma cell leukaemia is a rare type of (lymphatic) leukaemia in which many plasma cells are present in the blood, and in which the haemopoietic tissues are infiltrated with plasma cells at autopsy. Transitions occur to the localized plasmacytomata seen in multiple myelomata (Osgood, 1934).

Megakaryocytic leukaemia.—A pathological hyperplasia of the megakaryocytes, analogous to a leukaemia, has been reported in a few cases.

### DISEASES ALLIED TO OR RESEMBLING LEUKAEMIA

Pseudoleukaemia is an inexact and undesirable term loosely applied to any condition superficially resembling lymphatic leukaemia, but not showing leukaemic changes in the blood.

Lymphoblastoma is a term applied to a group of diseases characterized by an abnormal hyperplasia of the lymphoid tissue, with enlargement of the spleen and lymph glands. It includes lymphatic leukaemia (both in the leukaemic and aleukaemic stages), lymphosarcoma, and Hodgkin's disease, but not the infectious granulomata or ordinary neoplasms.



Cells from case of acute monocytic leukaemia. I to 15, monocytes. I and 3, monoblasts. I6 to 20, plasma cells.



Hodgkin's Disease (Malignant Granuloma).—This is a chronic disease of unknown etiology, possibly an infection, characterized by an infiltration of the lymphoid tissues and spleen (and occasionally the marrow) with a peculiar type of granulation tissue rich in epithelioid cells and giant cells (derived from monocytes, Sabin) and eosinophiles, which proceeds to extensive fibrosis in the advanced stages. The process starts in some single aggregation of lymphoid tissue, most often in the upper posterior cervical or supraclavicular glands on one side, or in the mediastinal glands, less often in the deep abdominal glands. It spreads first to the adjacent groups of glands, and secondarily to more distant groups. The spleen usually becomes enlarged. The glands remain discrete, but often form large masses which cause grave disturbances from pressure on neighboring structures. Bone lesions occur in 10% to 15% of the cases. The lung may be invaded. Intense pruritus, which may precede evident glandular enlargement, is a characteristic clinical symptom, as (in some cases) are recurring paroxysms of high fever (Pel-Ebstein), prostration, emaciation, and progressive anaemia, alternating with periods of normal temperature and relative well being.

The *blood* is never leukaemic. There may be a neutrophilic leukocytosis. In the early stages there may be an increase in monocytes and in platelets, and occasionally an eosinophilia, which is sometimes high. There is no lymphocytosis, and in the late stages the lymphocytes may be reduced. A severe anaemia gradually develops, which is somewhat hypochromic and microcytic in type. Biopsy is necessary for a positive diagnosis.

In the early stages deep radiation nearly always causes the glandular masses to disappear, with marked clinical improvement. Radiation soon loses its effectiveness, and death always follows, usually within two to five years.

Gordon (1933) has reported the production of a peculiar type of encephalopathy in rabbits following combined intramuscular and intracerebral injections of emulsions of enlarged lymph glands from patients with Hodgkin's disease, but not with other diseases such as lymphosarcoma or leukaemia. He believes the active agent is a filtrable virus, and he regards the test as specific and of practical diagnostic value. Opinion on the subject is divided, and further study is required to determine the validity of these views.

Lymphosarcoma (Kundrat) is a peculiar type of malignant tumor arising from lymphocytes. It starts locally, most often in the cervical or mediastinal glands, occasionally in the abdomen, and spreads both by direct extension and by metastasis to neighboring groups of glands. Late in the disease there may be metastases to other tissues, but the growth

tends to ensheathe rather than invade the neighboring organs. Enlargement of the liver and spleen is rare except in those cases in which the involvement is primarily abdominal. Growth is often rapid, and the glands tend to coalesce and form large tumor masses. A practically complete temporary remission usually follows deep radiation.

As a rule the *blood* is not leukaemic, and the lymphocytes are not increased. In a few cases, however, in the terminal stages there is an invasion of the blood stream by the pathological cells (*leukosarcoma*). There is an anaemia and usually a moderate neutrophilic leukocytosis. Biopsy is required for a positive diagnosis, but in the early stages differentiation from leukopenic lymphatic leukaemia may be difficult.

Myeloma is a malignant neoplasm arising from any of the parenchymal cells of the marrow (myelocytes, myeloblasts, lymphocytes, plasma cells, erythroblasts). The disease is focal, not diffuse, but multiple nodules develop, usually in many bones. These may give rise to palpable swellings, or to erosions of the bone leading to spontaneous fractures, and may cause violent pains and paralyses from pressure on the spinal cord or nerve roots. The bones of the trunk are chiefly involved. There may be metastases to other organs in the late stages.

A severe anaemia develops, usually classed as myelophthisic, and occasionally accompanied by an erythroblastosis and stimulation myelocytosis. The blood is not leukaemic, and the pathological cells rarely enter the circulation, although in a few cases of plasma-cell myeloma they have done so. The frequent (but not invariable) occurrence of Bence-Jones protein in the urine is of great diagnostic value (see p. 719).

Gaucher's disease is a congenital, often familial, but not directly hereditary constitutional anomaly of metabolism. It is characterized by a progressive hyperplasia of the reticulum cells of the spleen, liver, bone marrow, and, to a less extent, the lymph glands, the cells of which become stuffed with granules of the cerebroside kerasin. The disease is rare, and a majority of the cases have been in Jews. It begins in infancy and runs a slow chronic course, with an average duration of about 20 years, although some patients have survived to fifty or sixty. Clinically there is gross enlargement of the liver and spleen, brownish pigmentation of the exposed skin, and in the older cases often wedge-shaped, yellowish swellings (pingueculae) of the bulbar conjunctivae. Bone lesions are common. There may be bone pains, with deformities or pathological fractures, but more often the lesions can be made out only by means of roentgenograms. The cortex is thinned, the bone widened, while irregular areas of osteoporosis and increased condensation occur in the spongy bone. The first changes are said to occur in the distal end of the femur (Welt et al., 1929).

The blood shows a leukopenia and a diminution of platelets which may cause a secondary purpura haemorrhagica. An anaemia may develop, but is not constant. The differential count is normal. Diagnosis depends upon finding the characteristic "foum" cells in material obtained from the spleen or marrow by incision or puncture. (The superficial lymph glands do not usually show them.) These cells are very large, with a relatively small, pale-staining nucleus, and abundant cytoplasm which has a somewhat spongy texture, and shows a fibrillar structure in suitably stained preparations.

Splenectomy may relieve some symptoms, including the thrombocytopenia and bleeding, but it is not curative.

Nieman-Pick's disease is a similar metabolic disturbance in which phosphatides are stored in the reticuloendothelial cells. This disease, which is rarer than the preceding, is more acute, all dying during the first two years of life. Most of the cases have been in Jews, and many of them have also shown the amaurotic family idiocy of Sachs.

Schüller-Christian's disease, the third member of the group, is the result of a disturbance of the metabolism of cholesterol. This substance is stored (as fatty acid esters) in reticulum cells, which undergo a similar extensive hyperplasia. The disease is seen chiefly in young children, as it is usually fatal within a few years. Clinically it is characterized by the development of circumscribed bone defects, most often in the flat bones, particularly of the skull, which are eroded by localized yellowish nodules (xanthomata) of hyperplastic tissue arising from the periosteum or dura. Similar nodules may be found in the skin and serous membranes. Common secondary symptoms are exophthalmos and diabetes insipidus (from pressure of nodules on the hypothalamic region). There is also diffuse hyperplasia of reticulum cells in the marrow, liver, and spleen (although these organs are only slightly enlarged), and in the lungs and other organs. The early lesions contain large numbers of typical "foam" cells. In the late stage fibrosis occurs. Extensive pulmonary fibrosis may be the immediate cause of death.

The blood shows no characteristic changes. In late stages there may be a grave anaemia of the myelophthisic type, and a thrombocytopenia with purpura. In some cases there has been a leukocytosis, with a moderate monocytosis. The blood cholesterol is often high, but has not been uniformly so.

Foam cells of the same type have been found in the spleen in some cases of diabetes and "nephrosis," in association with high blood fat and cholesterol.

Infectious mononucleosis (glandular fever) is a benign infectious disease of unknown etiology which bears a superficial resemblance to acute leukaemia, both in its clinical features and in the lymphocytosis which accompanies it. It occurs chiefly in children or young adults, both as sporadic cases and in epidemics. Clinically it is characterized by an acute onset with fever, usually sore throat, marked swelling of the cervical lymph glands, a general glandular enlargement of variable degree, and often enlargement of the spleen. The cervical glands may become huge. Occasionally there is a complicating Vincent's infection. Recovery practically always occurs within a few weeks, but the glandular enlargement and the lymphocytosis may persist for months.

The total leukocyte count is usually from 10,000 to 20,000, with a lymphocytosis of from 50% to 90%. In some cases there is a normal total count, or there may be a leukopenia. The lymphocytosis may not reach its peak until after 1 or 2 weeks. The characteristic feature of the blood is the large number of pathological lymphocytes present. These are mainly large cells with abundant, usually basophilic cytoplasm which shows a spongy or foamy texture and usually contains numerous azure granules. Most of the cells show a pale perinuclear zone. The nucleus is often indented or partly lobulated.

It is usually mature in type, and shows a coarse chromatin network of thick strands and masses which may be quite dense, like plasma cells. Some typical plasma cells are often present. In some cases there are a few lymphoblasts, with nuclei showing a fine chromatin structure and nucleoli, and we have even seen cells in mitosis, but immature cells are not present in large numbers, as in acute lymphatic leukemia (see also Downey, 1922, 1935). The granulocytes are reduced in absolute number, and show toxic degenerative changes. The monocytes are diminished (or absent, Doan). The red cells and platelets are not altered, and there is no tendency to bleed. The lymph glands show hyperplasia, with proliferation of the peculiar lymphocytes, which often obliterates the normal architecture of the gland, but is less extensive and more "patchy" than in leukaemia. The unusual leukocytic response is definitely due to the peculiar stimulus exerted by the infectious agent, and not to a constitutional abnormality of the patient.

The serum in most cases, after 7 to 10 days, shows an increase in agglutinin for sheep red blood cells (Paul and Bunnell, 1932).

Method.—(1) Set up a series of 12 small test tubes. (2) In tubes (1) and (2) put 0.5 cc. of the serum to be tested, which has been inactivated by heating to 56°C. for 20 minutes. (3) To tubes (2) to (12) inclusive add 0.5 cc. of salt solution. (4) Prepare a series of dilutions, from I to 2 to I to 2048, by transferring 0.5 cc. of the mixture in tube (2) to tube (3) and mixing, and so successively to tube (12). (5) To each tube add 0.5 cc. of a 2% suspension of sheep corpuscles, washed as in preparation for a Wassermann reaction. (6) Add I cc. of salt solution to each tube. (7) Shake, put in the waterbath at 38°C. for one hour, and in the ice box over night. (8) Shake gently to resuspend the sedimented cells, and note the highest dilution in which definite macroscopic agglutination occurs, recording roughly the degree of agglutination in each tube.

In normal serum the titer is usually I-8 or I-16 (the first 5 tubes), rarely higher. In infectious mononucleosis it usually rises to much higher figures (occasionally to I-4000), although several examinations at intervals of a few days may be necessary to detect it. A rise in titer has been observed in individuals who have received injections of horse serum, but it is rare in other conditions. The titer is reduced (less than I-4) in leukaemia (Bernstein, 1934).

Recent work of Bailey and Raffel (1935) indicates that this agglutinin is not, as originally assumed, the heterophile agglutinin of Forssman, which is that present in the serum of normal individuals and of patients who have received horse serum. They find that, unlike heterophile agglutinin, it is absorbed by autoclaved ox cells but not by a suspension of guinea pig kidney. Therefore, if a positive reaction is obtained by the method cutlined, additional specimens of serum should be absorbed, one with ox cells and one with guinea pig kidney, and the titration repeated with each specimen. A positive reaction is indicated by the disappearance of agglutinin in the serum extracted with autoclaved ox cells, and by its persistence in that extracted with guinea pig kidney.

Agranulocytic Angina (Malignant Neutropenia).—This is a clinical syndrome of unknown etiology occurring chiefly in women, characterized by an acute onset with fever, prostration, ulcerative stomatitis or pharyngitis, an extreme neutrophilic leukopenia, and usually a rapidly progressive course and a fatal termination. Clinically the condition suggests a fulminant septic infection, but blood cultures are negative, or show organisms which can only be secondary or terminal invaders. The tissues do not show pyaemic lesions, and there is a striking absence of leukocytic infiltra-

#### MALIGNANT NEUTROPENIA

Vincent's organisms are frequently (but not constantly) present in the mouth lesions.

The blood shows a marked (or rapidly progressive) leukopenia, primarily of the granulocytes, which may entirely disappear. The total count is usually under 2000 and may fall to 100. The red cells and platelets are usually unaltered, and bleeding rarely occurs. There is no stimulation of the lymphocytes such as occurs in infectious

DIFFERENTIATION OF THE ANAEMIAS BY THE BLOOD PICTURE

	Haemoglobin	Red cells	Color index	Volume index	Anisocytosis	Poikilocytosis	Macrocytes	Microcytes	Color of cells	Polychromatophilia	Reticulocytes	Nucleated red cells	leterns index	Urobilinuria	Leukocyte count	Platelets	Gastric acidity	Response to iron	Response to liver	Splenomegaly
Acute posthaemorrhagic	50- 80	3.0- 4.5	N- L	N-	+	o	0	=	N- P	+	+	±	×	o	Н	н	×		o	Ü
Chronic post meet carbacter	60	2.0-	L	L	+2		Ξ	+	P	Ŧ	Ŧ	Ξ	F.	0	H	H.	N	Ŧ	0	0
Idiopathic hypochromic	20- 60	3.5- 4.5	L	L	+	±	=	Ŧ		0	Z	0	L	0	Y.	Z	o	÷2	0	Ŧ
Hookworm	30- 70	4.5	L	Ĺ	Ŧ	±	0	+	P	0	<u>N</u> -	0	N-	٥	7	N	V	+	0	0
Cancer of stomach	20- 80	2.0- 4.5	L	L	+	±	0	+	P	=	τ.	0	N	0	H		0	-	0	0
Chlorosis	20- 60	3.5- 4.5	L	L	±	0	0	T	P	0	0	0	L	0	Z		7.	+ 1	0	o
Chronic infection, severe.	30- 70	2.5- 4.5	L	L	+	0	0		P	0	0		Ţ.	0	T	<u> </u>	7.	0	0	Ξ
Malaria	70	4.0	N	N	+	± 1	=	=	Z,	Τ	Ŧ.	0	H	+	L	V	N.	0	0	+
Pernicious, active stage	30- 70	3.0	Н	H	+3	+	2	7	 U	+			H	<u> </u>	L	L	0	Ú,	- 4	Ξ
Sprue: (1) macrocytic	70	2.0- 3.5	H	. i	-, -				P	=	N-	=	7.	±	L	v	- N			0
(2) hypochromic	30- 70	3.0- 4.5	L N	L	+	+	_	+	N	0		0	Z	0	L	L	<u>Z</u>	_	0	0
Erythroblastic	30- 60 30-	1.5- 3.5 1.5-		N	± +	±	о —	• ±	<u>Z</u>	° +	0 —-	0 <del>+</del> 3	~	0	H	H	7	<u> </u>	0	
Haemolytic	80	4.5 1.0-	N-7.	N	+	-0	=		7	_	<u>_</u>	Ŧ,	H	+	N-	H	<u>_</u>	0	0	王
Familial haemolytic jaun-	80	4.0 I.5-	<u>N</u> -	<u>Z</u> -	+	-	- : U	- :	Ð				rī.		H	7.			0	_
dice. Sickle cell	70	4.0	H	$\frac{H}{N}$	+2	<u>+ 2</u>		+	<u>Z-</u>	<del>-</del> 2	==	72	Ħ	-	H	<u>N</u> -	N	0	0	-
Lead poisoning	50-	4·5 3·0-	Ž-	<u>Z</u> -	- <u>+</u>	o	0	±	7.	72	Ţ-2	+	<u>N</u> -	±	H	H	7.	0	0	0
Banti's disease	25-	2.0-	L	는	=	+	0	+	P	0	0	0	품	- <del>-</del>	L	L	T	0	0	÷
Thrombopenic purpura	50~	3.5 2.5	N	N	0	0	o	0	N	±	0	ō	$\overline{\mathbf{X}}$	0	$\overline{\mathbf{v}}$	L-	Z	0	0	0
Acute leukaemia	20- 100	5.0 1.0-	N	N	+	±	±	+	N	+	Ŧ	+2	N	0	V	L-	N	0	0	Ŧ
	1 /4	, 5.0	1	1	1					l						1			l	

The data apply to very the construction whom the disease is well marked but not of maximum severity.

There are numerous the construction (see text).

D = Dark
H = High or increased.

Y = Variable.
+ = Present or increased.

L = Low or decreased.
N = Normal or not characteristically altered.
P = Pale.

<sup>+ =</sup> Present or increased. ± = Inconstant or sparse.

o = Absent or sparse.

mononucleosis. If the patient survives a week or more, granulocytes may reappear, and not rarely a few myelocytes and myeloblasts may be found, suggesting an acute leukopenic leukaemia. The sternal marrow (biopsy) at the height of the disease shows practically complete disappearance of granulocytes, without a proliferation of primitive cells such as is found in leukaemia.

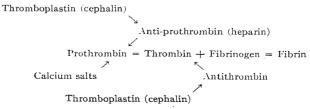
Recovery occasionally occurs spontaneously, or (probably more often) after nucleotide injections. In such cases a normal blood picture may be restored, but often a lesser degree of granulocytopenia persists. Such patients are prone to recurring acute attacks, any one of which may prove fatal. Milder cases with less marked granulocytopenia occur, and are probably more common than has been realized.

In a few cases an extreme leukopenia has been recognized shortly before the onset of the fever and symptoms of acute illness. This, with other facts, suggests that in this condition damage to the marrow, or a constitutionally defective marrow, is the primary factor, and that infection occurs secondarily, as a result of lowered resistance due to lack of leukocytes. There is a striking tendency to the development of ulcerative or necrotic lesions of the mouth in other diseases associated with a lack of granulocytes (as in acute leukaemia). That the marrow may be inherently defective is shown by the occurrence of individuals who show, over a period of years, either persistent or periodically recurring granulocytopenia, which may or may not be accompanied by active illness. Recently, however, emphasis has been placed on the use of amino pyrine and related drugs as a cause of the granulocytopenia. There is definite clinical and experimental evidence that aminopyrine tends to injure the marrow in this way. Such drugs should be rigidly withheld from patients with this tendency. However, the rarity of the condition, as contrasted with the widespread use of these drugs, indicates that individual predisposition plays a large part. Acetyl-salicylic acid and the simple barbiturates appear to have relatively little of this effect.

#### HAEMORRHAGIC DISEASES

By haemorrhagic disease is meant any condition in which there is a tendency to abnormal or protracted bleeding. Haemorrhages due to gross anatomical lesions are not included. The tendency to bleed may be due to disturbances of coagulation, or to abnormalities of the endothelium of the capillaries, or to both.

The following diagram illustrates a commonly accepted view as to the mechanism of normal coagulation in vitro. based largely on Howell's theory.



Normal coagulation in the body also involves the active participation of the *platelets*, which give to the thrombus its definite structure, and are essential for its proper retrac-

tion, which in turn is necessary to bring about effective closure of the vessels. They also serve as important sources of cephalin and prothrombin. A lack of platelets also appears to result in an injury to the capillary endothelium, which leads to the easy production of mechanical defects in the vessel wall, through which seepage of blood takes place.

Fibrinogen is formed in the liver. Haemorrhage due to lack of fibrinogen is met with in grave liver disease (acute yellow atrophy and poisoning with chloroform or phosphorus). It has also been reported as a rare congenital constitutional anomaly, fibrinopenia ("pseudohaemophilia"). It is associated with a prolonged bleeding and coagulation time, and scanty clot-formation.

Prothrombin deficiency has been reported in haemorrhagic disease of the new born. A deficiency of available (ionized) calcium as a cause of haemorrhage is rare. It was reported by Lee and Vincent (1915) as the cause of bleeding in chronic obstructive jaundice. There is moderate prolongation of the coagulation time, with a normal platelet count. Others have questioned this observation, and the mechanism of the bleeding is still unsettled.

Thus far there is no proof of a deficiency of cephalin, or an excess of heparin or antithrombin as a cause of bleeding.

A deficiency in blood platelets is the cause of bleeding in nearly all cases in which the latter is due to a disturbance of coagulation. This syndrome is known as thrombocytopenic purpura (purpura haemorrhagica). It is characterized clinically: (1) by spontaneous extravasations of blood into the skin and the mucous and serous membranes (purpura); and (2) by haemorrhages from the mucous membranes. The blood shows a normal coagulation time, but a protracted bleeding time, defective clot-retraction, and a positive capillary-resistance test. As a rule these changes are roughly parallel with the degree of platelet reduction. Bleeding usually appears if the platelet count falls below 50,000. There are exceptions, however, since bleeding may be observed with platelets at higher levels, and it may be absent or delayed with much lower counts. The bleeding is due to abnormalities in the endothelium of the small vessels which are associated with and probably caused by the reduction in platelets.

Thrombocytopenic purpura occurs: (1) as a symptom of a variety of diseases in which the activity of the marrow is depressed, the symptomatic thrombocytopenic purpuras (listed on p. 345); and (2) in the specific disease idiopathic (primary) thrombocytopenic purpura.

Idiopathic Thrombocytopenic Purpura.—This disease is probably dependent upon a constitutional abnormality of the bone marrow, and is sometimes hereditary. It affects both males and females. The disease usually appears in childhood, and runs a protracted chronic course, marked by recurring periods of profuse bleeding associated with a marked reduction in platelets, which may last from a few days to several weeks. These alternate with periods of remission of varying completeness and duration. As a rule symptoms subside, and the platelets rise above the critical level, although they often remain well below normal. More rarely the disease begins in an adult with an

#### HAEMORRHAGIC DISEASES

acute severe attack which may be quickly fatal. Complete permanent recovery may occur, or the usual chronic, relapsing type of the disease may follow. Bleeding occurs most often from the nose, gums, uterus, stomach, intestine, or urinary tract. A cerebral haemorrhage may occur, but bleeding into a joint is rare. Purpuric eruptions may be absent. For a time bleeding may be restricted to a single source, as from the uterus. There is no fever or other evidence of infection.

The blood at first shows no abnormality of the red cells or leukocytes, although there may be a moderate leukopenia. An ordinary acute posthaemorrhagic anaemia soon develops, and frequently a secondary leukocytosis. The individual platelets are often abnormal; there are some huge platelets, while many are minute.

Venom test.—Peck et al. (1936) have reported finding a positive reaction to intracutaneous injections of moccasin venom in cases of idiopathic thrombopenic purpura and certain drug purpuras during the period of active bleeding, with a disappearance of the reaction in cases which improved clinically. This was not necessarily associated with an increase in platelets or a reduction of the bleeding time. They regard the test as of prognostic as well as diagnostic value.

Procedure: Inject intracutaneously o.1 cc. of a 1 to 3000 dilution of standard venom (obtainable from Lederle Laboratories). A positive reaction is indicated by the appearance of an ecchymosis at the site of injection within one hour. An injection of salt solution is given as a control.

Splenectomy is usually followed by an abrupt cessation of bleeding and a critical rise in platelets to normal or above, which may be marked within a few minutes after the organ has been removed. This fact suggests that the disease may be due to a pernicious overactivity of the spleen, manifested either by an excessive destruction of platelets in the circulation, or an inhibition of their production. Although the platelet count usually falls again after a few months, as a rule bleeding does not recur. Some cases, however, have relapsed (accessory spleens?). Splenectomy is contraindicated in other types of purpura. Transfusions also stop bleeding, but only for 3 or 4 days. Other measures are very uncertain in their effect.

Purpuric eruptions due to abnormalities in the small vessels occur in a variety of diseases in which the platelets are normal in number, and bleeding is slight or absent. These include:

- 1. Intoxications with various drugs, many of which may cause a thrombocytopenia (in other patients). Many drug "idiosyncrasies" are included in this group.
- 2. Acute infections, such as smallpox, typhus fever, epidemic meningitis, sepsis, bacterial endocarditis.
  - 3. Senile and cachectic purpuras.
- 4. Scurvy (avitaminosis). Here the bleeding is chiefly under the periosteum or into the deep muscles of the legs. There are small purpuric spots about the hair follicles of the legs. The gums and mucous membranes do not bleed.
- 5. "Anaphylactoid purpura," which occurs chiefly in children and young adults, includes the following symptom complexes, which may occur singly, or in various combinations.
- (a) Cutaneous eruptions: erythaema multiforme, with urticaria and angioneurotic oedema, as well as purpura.
- (b) Schönlein's purpura, a mild arthritis, most often affecting the knees and ankles, and usually showing clusters of purpuric spots about the affected joints.
- (c) Henoch's purpura, characterized by recurring, acute, often severe attacks of colicky abdominal pain with tenderness and rigidity, vomiting and diarrhoea or con-

stipation, and often with some blood in the stools and vomitus. Bleeding is never profuse. Cutaneous eruptions are usually present. The attacks are accompanied by fever, slight leukocytosis, and symptoms of an acute infection. Haematuria or acute nephritis may follow. The condition has been mistaken for acute appendicitis, and operations needlessly performed. There is no disturbance of coagulation, and no anaemia. The capillary resistance test is variable, but usually negative. The disease resembles serum disease more than it does thrombocytopenic purpura. Differentiation from the latter is important, because transfusion and splenectomy are contraindicated.

Haemophilia.—This is a very rare, hereditary disease due, probably, to a constitutional abnormality of the platelets. It is characterized by a tendency to protracted bleeding from slight cuts and bruises. The bleeding often persists for days or even weeks as a slow oozing which may eventually be exsanguinating. Bleeding often occurs also into the subcutaneous tissues and into the joints, but rarely from the mucous membranes, except from the nose or gums. Many victims have died from haemorrhage following circumcision or tooth extraction. The tendency to bleed is always manifested in infancy (although rarely during the first week), and the severer cases usually die during the first year. The tendency to bleed varies markedly at different times in the same individual.

Bleeding does not occur from a simple pin prick, and venepuncture can be performed without danger. The bleeding time is usually normal, and the capillary resistance test is negative. Purpuric eruptions never occur. The coagulation time and "prothrombin time" are greatly prolonged, often to an hour or more. However, this varies greatly in the same individual on successive examinations, and at times may be nearly normal. The clot retracts normally after it once forms. The platelets are normal in number, but are abnormally resistant, and do not disintegrate and initiate coagulation as do normal platelets. The blood is otherwise normal, or shows the changes of an ordinary posthaemorrhagic anaemia.

The bleeding may be checked for 3 or 4 days by transfusion.

The disease occurs only in males, and direct transmission is solely through females, the conductors, as a recessive, sex-linked character. The sons of a haemophilic male never have the disease, and never transmit it, but half his daughters become conductors. Of the children of a conductor, half her sons manifest the disease, and half her daughters become conductors, and transmit the defect. Theoretically all the children male and female, of a haemophilic male and a conductor should have the disease, but thus far no such cases have been observed.

Haemorrhagic disease of the new born (melaena neonatorum) is a disease of unknown etiology occurring in infants during the first week of life, and characterized by a marked tendency to bleed. It is not hereditary, and the sexes are equally involved. The bleeding most often occurs as: (1) cutaneous haemorrhages, either oozing from abrasions, or large haematomata; (2) bleeding from the umbilicus; (3) bleeding from the intestine, causing black tarry stools (both rare in haemophilia); and (4) cerebral haemorrhages, which are often fatal, or permanently crippling. Bleeding is often exsanguinating, and the mortality of untreated cases is from 60% to 80%. Transfusion stops the bleeding promptly. Recovery is complete and permanent. The nature of the coagulation defect has not been adequately studied. Scanty observations indicate a prolonged bleeding time and coagulation time, but a normal platelet count. A deficiency of prothrombin has been described.

For other rare types of haemorrhagic disease, see Christian, in Oxford Medicine.

# PART III ANIMAL PARASITOLOGY

#### CHAPTER XVI

# GENERAL CONSIDERATIONS OF CLASSIFICATION AND METHODS

Animals that are in all respects alike we term a species. Of course the male and female of a species may be very unlike, but as a result of mating they produce young having characteristics similar to the parents. Now if, as in the case of the mosquitoes, we find some with straight silvery lines and others uniformly showing crescentic silvery bands about thorax, yet resembling each other closely in the respect of being small, dark, brilliantly marked mosquitoes, we should consider them as being separate species with a certain relationship to which the term genus is applied.

The term "genus" is of wider application than the word "species." Thus animals which agree in the main characteristics of size, proportion of parts, and general structure are placed in the same genus.

In naming a species we always first write the name of the genus which has a Greek or Latin name, commencing with a capital, and follow with the specific name, which latter commences with a small letter. Thus we designate the common tropical house mosquito that is active at night, Culex quinquefasciatus. This species is characterized by the failure of the abdominal bands to reach the sides, especially posteriorly, thus differentiating it from a closely related species in colder climates, Culex pipiens, in which species the abdominal bands reach the sides.

For the generic name to be valid it must be one that has not already been given to another group of animals. The definition of a genus is based on the first described species of the genus, which is designated the type species or genotype, and if at any time such a genus is broken up into other genera this genotype remains with the original genus. When a valid name has been given a species, and that species is later on transferred to another genus, the specific cognomen must attach to the new genus; thus Culex aegypti when placed in the genus Stegomyia becomes S. aegypti and when placed in the genus Aedes becomes A. aegypti.

The specific name may be a noun in the genitive. If an adjective it must agree in gender with the generic name.

It is permissible to have a masculine noun as a specific name with a feminine generic

If the specific name is a modern patronymic we add i in the case of a man or ae for a woman to the exact and complete name of the person.

Again certain genera show resemblances which enable us to make broader groupings to which we apply the term tribe. Thus the genera Aedex and Culex have certain similar larval characteristics and the imagos a nude metanotum; we therefore classify all species of these genera under the designation Culicini. The name of a tribe ends in "ini." Now, again, certain insects are different from others in having a long proboscis adapted for piercing. We find that not only do the Culicini have such characteristics but the same is observed with the Sabethini. These we term a subfamily and we speak of the Culicinae, meaning the true mosquitoes. The name of a subfamily ends in "inae." Again, certain insects differ from others in having scales on their two wings. Thus we find that not only do the Culicinae (true mosquitoes) have such characteristics but also the Corethrinae. These together we term a family and speak of them as the Culicidae. The name of a family ends in "idae," this suffix being added to the inflexional stem of the name of the first named genus of the family. Many families are not subdivided into subfamilies but are directly separated into genera. Again a genus may have only a single species.

At times a family may be raised to *superfamily* rank—the subfamilies then becoming families. Thus the families Ixodidae and Argasidae belong to the superfamily Ixodoidea. The termination for a superfamily is "oidea."

When there are a number of families agreeing closely in some striking characteristic, we group them together into an *order;* thus, the family of mosquitoes closely resembling many other families of insects in possessing a pair of well developed wings is grouped in the order Diptera, all of which resemble certain other animals in the possession of a distinct head, thorax and abdomen with three pairs of legs projecting from the thorax. This collection of animals we call a *class;* thus, we speak of the class Insecta. It will be observed that the insects have no internal skeleton, but instead a chitinous cuticle, the exoskeleton. Spiders, ticks, etc., resemble them in this respect, and we now apply to all such animals the wider designation, *branch* or *phylum* Arthropoda.

Inasmuch as the animal kingdom is divided into the branches Protozoa, Porifera, Cnidaria, Echinodermata, Vermes, Annelida, Arthropoda, Mollusca and Chordata, we see that the branch is the largest grouping we employ. To descend in the scale we have belonging to the branch, the classes; to the class, the orders; to the order, the families; to the family, the subfamilies; to the subfamily, the tribes; to the tribe, the genera; to the genus, the species. Occasionally a species is further divided into subspecies.

By a type species we understand the species of a genus always referred to as representing the genus.

Many favor alliteration for type species, as Heterophyes heterophyes.

The male animal is designated by the sign of Mars ( $\varnothing$ ), the female by that of Venus ( $\diamondsuit$ ).

# CLASSIFICATION OF ANIMAL PARASITES (According to Stiles)

- 2. Body more or less flattened dorso-ventrally..... 4

#### GENERAL CONSIDERATIONS OF ANIMAL PARASITISM

While it is usually comparatively easy to determine whether a parasite be vegetable (bacteria, moulds) or animal, yet there are many parasites about which a doubt exists as to their true position. This is particularly true of the group of spirochaetal organisms causing such important diseases as syphilis, yaws, relapsing fevers, rat-bite fever, and infectious jaundice. This group of organisms has been removed from Part III to Part I, although there are many protozoologists who still lay claim to these parasites. Those filtrable virus diseases showing inclusion bodies (Chlamydozoa) were formerly grouped with the animal parasite section, but are now considered in Part I. Rickettsias, most probably, are closely related to bacteria, as is also Bartonella.

As noted in the preceding table of phyla the animal parasites of man belong to protozoal, helminthological and arthropod branches. Among protozoal and arthropod animals the free-living forms make up a large proportion of such branches, but with the helminths the state of parasitism is almost universal. An exception to this is the class Turbellaria (planarians) which, in contrast with the cestodes, trematodes, and nematodes, are free-living, usually found swimming about in salt or fresh water or occasionally found in moist earth.

The more adapted to parasitism an animal becomes the more striking the change in structure from that characterizing free-living forms. In particular does the locomotor apparatus degenerate, and frequently it is replaced by fixation organs, such as suckers or hooklets. At the same time the muscular and nervous systems undergo degeneration, and with the entozoal parasites the furnishing by the body fluids or tissues of the host of suitable food for the parasite brings about simplification or disappearance of the digestive apparatus. So great may be the degeneration of structural parts that certain parasites, as the tongue-worms (Linguatulida), which are arachnids, resemble tape-worms more than they do the mites and ticks to which they have a classificatory relation.

When we consider the reproductive system of obligate parasites we find extraordinary development. It will be evident that should the entire life cycle of an animal
parasite be passed in a single animal the continuation of the species would be endangered
—the parasites would die when the host ceased to live. For this reason the transfer
of the parasite to another individual is necessary, or a substitution of hosts, or adoption
of free-living periods. The chances of finding suitable intermediary hosts and of a
return to the original one must be very slight for an individual, and to provide against
possibilities of extinction there is prodigality in reproduction. In the case of the lung
fluke and certain other flukes three hosts are connected with the life history of the parasite. Such animals as flukes and tape-worms seem to be made up largely of sexual
organs and accumulations of eggs. The mature segments of Hymenolepis nana show
solely a mass of eggs.

Parasites damage the human host in various ways. Some consume the food supply in the alimentary tract to such an extent that malnutrition may result. Others actually destroy important tissues or organs. There may be such a loss of blood by the wounding of blood vessels, incident to the wanderings of the parasite, or in satisfaction of food demands, that marked anaemia results. Some parasites elaborate toxic material. There is also the probability that the traumatism to intestinal or other structures may provide an atrium for bacterial infection.

At times we divide parasites into ectoparasites and endoparasites, according as they live upon or within the body of the host. It would be an easy matter to decide that the body louse belonged to the first group and the hookworm to the latter, but with the itch mite, which penetrates the skin, or the chigoe, which burrows under the toe nail, it would be a matter of difficulty.

It is usual to use the term infection for parasitization within the body, as a flagellate infection, and infestation for that on the surface of the body, as an infestation with

Acarina

parasites; some burrow under the skin or live in the hair follicles; acarines......

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certain rules governing the naming of animals. Of these, the law of priority provides that the oldest published name, under the code, of any genus or species is its proper zoological name. The name must appear in a well recognized publication and even the printer's proof does not establish priority. The history of the naming of the organism of syphilis illustrates this well.

The law of priority and the name of the cause of syphilis.—Schaudinn gave this organism in 1905 the name of Spirochaeta pallida. Ehrenburg, in 1834, had used the name Spirochaeta for protista of a different character, so that this designation of the genus was not permissible under the code. Later on in 1905 Vuillemin proposed the generic name Spironema. This name, however, was found to have been used in 1864 by Meek for a genus of mollusks and by Klebs in 1892 for a genus of flagellates. Consequently, being a homonym, it was not available.

(A generic name can be applied to only one animal genus, and if a similar name is subsequently given another genus it is a homonym and is to be rejected.)

On Dec. 2, 1905, Stiles and Pfender then proposed the name *Microspironema* but as Schaudinn published on Oct. 26, 1905, the designation *Treponema*, the name *Treponema* pallidum had to be accepted as the proper zoological name for the organism of syphilis.

The question of the name of the organism of relapsing fever.—The generic name Spirochaeta not being available, Noguchi, in 1918, proposed the name of Spironema. As noted above this name was not valid. In November, 1907, Sambon proposed the name Spiroschaudinnia for the blood spirochaetes and this name would seem the proper one were it not for the fact that in July, 1907, Swellengrebel proposed the name Borrelia for the group of organisms of which B. gallinarum was the genotype. Sambon's classification in which he used Spiroschaudinnia for blood spirochaetes was presented in a paper read before the British Medical Association in July, 1907, but as it was not published until November, 1907, this is the time of priority. It is the publication in an acceptable journal, and not the time a paper is read, that gives basis for priority. Swellengrebel differentiated Borrelia from Treponema on the basis of peritrichal flagella for the spirochaetes of the blood—a conception which has not been confirmed. however, does not invalidate the name because the International Commission holds that we name the objects themselves, not our conception of said objects, and Swellengrebel named spirochaetes, of which gallinarum was the genotype, as Borrelia. Even if Borrelia could be rejected on the ground of an erroneous definition the same would hold true for Spiroschaudinnia which Sambon stated to form sporozoites—also an error in conception.

The name of the mosquito transmitting yellow fever.—The name of the yellow fever mosquito, so long established as Stegomyia fasciata, has as a consequence of the operation of the law of priority been changed several times during the last few years, older names designating the species having been found in the literature. The generic name Culex which in former times was used for the entire mosquito family was replaced in 1901 (Theobald) by the generic name Stegomyia. Recent authorities state, however, that the generic characteristics of Stegomyia are not distinctive enough to warrant the formation of a separate genus, and for this reason the generic name Stegomyia was dropped and replaced by Aedes, a reconstructed genus that included amongst others the various species of Stegomyia. At present, however, there is shown a marked tendency to subdivide the genus Aedes into several subgenera, one of which is Stegomyia, so that the proper generic name for the yellow fever mosquito then would be Aedes (Stegomyia).

The specific name of this mosquito presents a more varied history. In all works on mosquitoes a large number of synonyms are given for this species, some of which are, albopal posis, fasciala, persistans, nigeria, elegans, bancrofti, excitans, etc.; but these various names have all been dropped because the species had been described and named prior to their introduction. Up to a few years ago discussions of this mosquito considered only two names, namely fasciata and calopus, Fabricius having named the species, Culex fasciatus in 1805 and Meigen C. calopus in 1818. Priority indicated that fasciatus should be the specific name but according to some authorities this name was preoccupied (Müller, 1764), thus making calopus the valid name. Further investigation has shown, however, that the claims for preoccupation of fasciata were not well founded, and hence this name would be the proper one, if it were not for some recent findings which show that Poiret named the species, C. argenteus in 1787 and Linnaeus, C. aegypti in 1762. The descriptions of the mosquito given by these men undoubtedly indicate that they were dealing with the species in question and hence, according to the law of priority, the name given by Linnaeus is the proper one.

Thus the name of the yellow fever mosquito as established today is Aedes (Stegomyia) acgypti Linnaeus.

Of unusual interest is the question of the name of the old-world hookworm. Dubini, in 1843, named a nematode found by him in man Agchylostoma. By the law of priority this spelling would have been the correct one had he not stated in a footnote that the generic name was derived from two Greek words  $\alpha\gamma\chi\dot{\nu}\lambda\sigma\sigma$  and  $\sigma\tau\dot{\nu}\mu\alpha$ . Having indicated the origin of the name it became subject to the rules for correct transliteration, which is Ancylostoma.

In case of larva and adult, or male and female, formerly considered different animals, but subsequently found to be the same, the oldest available name becomes the name of the species.

Another point is that names are not definitions, consequently the fact of lack of appropriateness of any name is no objection to its continuation. This will appeal to anyone as a wise provision, because if a different name were substituted each time a designation more descriptive or applicable was invented it would be utterly destructive to system. When it is considered that some of our parasites have approximately fifty different designations, for the most part given by medical observers, it will be appreciated how much the zoologist has aided us in trying to eliminate all but the single proper zoological name.

It is a rule of zoological nomenclature that zoological names are independent of botanical ones, so that the prior use of a generic name for a plant is not a valid objection to its use for an animal, but it is well to avoid introducing into zoology as generic names such as are already in use in botany.

The objections so frequently heard among physicians in connection with adopting new names for old ones are not well founded. Wherever confusion has reigned, the establishment of order always results in temporary greater confusion. There is no doubt that the student taking up this subject a few years hence will have the satisfaction, thanks to the zoologist, of having to burden his mind with only one name for

each parasite. There is only one correct name for an animal and all other names are synonyms. The principal cause of changes of names is that our conception of the relationships of animals changes.

6. Terminology.—This applies to appropriate designations for different organs, symptoms, etc., and is not subject to any rule other than that of good usage.

Thus the terms cirrus in the case of male copulatory organ of flukes, spicule for the same in nematodes and penis, in connection with insects, would be instances of terminology.

- 7. Pseudoparasitism.—Where organisms enter the body accidentally, and when such sojourn in the body of man plays no part in the life history of the organism, we employ the term pseudoparasitism. For example: Fly larvae swallowed by man and passed out in the faeces. We also use the terms temporary parasites (bedbug) and permanent parasites (liver fluke).
- 8. Hosts.—The animal in which a parasite undergoes its sexual life is called the definitive or final host, that in which it passes its larval existence the intermediate host. For example: Man is the intermediate host of the malarial parasite, the mosquito the definitive host. A single animal may, however, be both definitive and intermediate host; thus, *Trichinella* may pass its larval existence in the muscles of man and its sexual life in his intestines. With certain infections we have two intermediate hosts, as in paragonimiasis, where the first intermediate host is a mollusk and the second intermediate one a crab.
- 9. Metaxeny.—This is a term recently introduced to express the conditioned existence of a parasite upon an intermediate host. Examples are furnished in the dependence of malarial parasites on the human host and of the schistosomata on mollusks.
- ro. Heredity, Congenitalism.—Hereditary characteristics are those which were present potentially in the ovum or spermatozoon before fertilization; congenital ones those which originate after fertilization, but before birth. South African tick fever is probably an instance of heredity, the spirochaetes having been found in the ovary and ova of the female tick.
- 11. Heterogenesis, Parthenogenesis.—Offspring differs from parent, but after one or more generations there is reversion to the parent form.

Strictly speaking, the term heterogony applies to reproduction when a sexual generation alternates with a parthenogenetic one. Where a non-sexual generation, as by division or budding, alternates with a sexual one, the process is called metagenesis. In parthenogenetic reproduction, eggs develop without fertilization by spermatozoa.

In coccidiosis we have a sexual cycle (sporogony) alternating with a non-sexual one (schizogony). In the infection with *Strongyloides* we have a sexual cycle alternating with a parthenogenetic one. In malaria we have a sexual generation and a non-sexual one.

- 12. Homology and Analogy.—By homology we understand the anatomical correspondence of the organ of one animal to that of another. Thus the fore leg of a quadruped and the wing of a bird are homologous organs. Analogy refers to physiological or functional agreement; thus the lungs of mammals and gills of fish, both with respiratory functions, are analogous organs. The first trace or appearance of an organ in an embryo is known as the anlage of the organ.
- 13. Protista.—Haeckel proposed this name for unicellular animals and plants, thus including protozoans and protophytes in a kingdom separate from the animal and vegetable kingdoms. We have sufficient difficulty in drawing the line between an

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animal and vegetable organism and to make a demarcation of a new kingdom from the two usually recognized would add to our difficulties.

14. Phylogeny and Ontogeny.—Phylogeny deals with the evolution of a group of animals. The phylogenetic or ancestral history of the genesis of the horse of the present day shows that it developed from an animal with four toes on the fore foot and three on the hind foot. Ontogeny deals with the evolution or germ history of an individual and with the development from egg to mature adult.

#### EPIDEMIOLOGICAL CONSIDERATIONS

Some apply the term intermediary host to one in which the parasite does not undergo development and restrict intermediate host to one in which a non-sexual cycle takes place. It is probable that the definitive host is the original host of the parasite and as a rule such a host does not directly suffer from the harboring of the parasite. When a parasite leaves its definitive host it frequently assumes a resistant stage and as such may be taken up by another host and continue as such in this second host, without multiplying or producing ill effects in such second host. This parasite, which is simply protected by the host, and does a host no damage, is an example of commensalism. This is exemplified in the carriage of endamoebic cysts by the house fly. The cysts leave the human alimentary tract as such and after a period of residence in the house fly leave this protective host, in its faeces, to be subsequently ingested by man and resume the developmental life which causes dysentery. With the parasites which undergo a non-sexual multiplication in the second host we have an instance of a true intermediate host and as a rule such a host is damaged by the parasite. For instance, Plus modium vivax produces disease in its non-sexual life in man, its intermediate host, but apparently does no harm to its definitive host, the anopheline mosquito, while undergoing its sexual cycle. In the same way Wuchereria (Filaria) bancrofti does not directly harm man, its definitive host, but when undergoing its non-sexual life in the culicine mosquito it is inimical to the life of the mosquito. While, as a rule, no harm is done man by the filarial infection and, as is well known, the peripheral blood of a human host may teem with filarial embryos without his showing any ill effects, yet with secondary factors (pyogenic organisms producing lymphangitis or effects of lymphatic obstruction) we may have clinical manifestations of disease.

Transmission of Parasites.—Where the definitive and intermediate hosts are both vertebrates, the passage from one to the other is ingestive; thus cattle take in the onchosphere of Tacnia saginata at the time of grazing and man subsequently eats the raw or insufficiently cooked meat of such cattle containing the embryonic parasite (Cysticercus hovis). When transmission is from a vertebrate to an arthropod the latter take the parasite into its alimentary tract so that the process is ingestive. With the transfer of a parasite from arthropod to vertebrate this may be ingestive, as with endamoebic cysts from the fly, but is more often inoculative, as when man is bitten by a transmitting tick, mosquito, or fly. When there is no change in the parasite, but simply a direct transfer from the biting parts of the fly, recently contaminated by feeding on one vertebrate, to another vertebrate, we have a direct inoculative transmission. This is recognized for certain other animals but probably does not occur in the case of man. When the parasite undergoes a developmental or cyclical change in the arthropod and is subsequently injected into man by the biting process we have an indirect inoculative transmission.

#### 396 GENERAL CONSIDERATIONS OF CLASSIFICATION AND METHODS

Reservoir of Virus.—This is a very convenient term to employ in discussing epidemiology. By this is understood the source from which a host becomes parasitized. A man having sexual malarial parasites in his blood is a reservoir of virus for the parasitization of anopheline mosquitoes which may take up his blood. In malaria, man is the only reservoir of virus, but in some diseases some other animal as well as man may be a reservoir of virus. It is considered that wild antelope may be the reservoir of virus for Trypanosoma rhodesiense as well as man. These reservoirs may provide material for the parasitization of either the definitive or the intermediate host.

# CHAPTER XVII

# TABLE OF IMPORTANT ANIMAL PARASITE DISEASES

DISEASES CAUSED BY PROTOZOA

Parasite	Defin. host	Intermediate host	Important reservoir of virus	Transmission and pathogenicity
Endamoeba histolytica.	Man.	Not required.	Man-carrier stage (faeces).	Cysts in food or water. Flies may act as carriers. Ingestive. Amoebic dysentery.
Balantidium coli.	Man (hogs).	Not required.	Man-carrier stage (hogs).	Transmission prob- ably same as for E. histolytica. Inges- tive. Balantidium dysentery. Anaemia.
Giardia 1 a m b l i a (Lamblia i ntestinalis).	Man.	Not required.	Man-carrier stage (mice and rats?).	Transmission probably same as for E. histolytica. Rat faeces on human food important. Ingestive. Lamblia dysentery. Giardiasis.
Trypanosoma g a m - biense and rhode- siense.	Fly (Glossina species).	Man.	Man—game animals? (blood).	Cyclical development in tsetse fly. Inocu- lative. Sleeping sick- ness.
Trypanosoma cruzi.	Triatoma megista and related insects.	Man.	Man.	Cyclical development in bug. Inoculative. Brazilian trypanoso- miasis.
Leishmania donovani, infantum, tropica and braziliensis.	Not surely known.	Man.	Man.	L. donovani—probably transmitted by various species of Phlebotomus—kalaazar. L. infantum—infantile leishmaniasis. L. tropica—Oriental sorc. L. braziliensis—A merican leishmaniasis.
Plasmodium malariae, vivax and falciparum.	Mosquito (Anophe- line spe- cies).	Man (with schizonts).	Man (blood) (with gametocytes).	Cyclical development in mosquito—12 days. Inoculative. Malaria.

DISEASES CAUSED BY PARASITES FORMERLY CONSIDERED PROTOZOA

Parasite	Defin. host	Intermediate host	Important reservoir of virus	Transmission and pathogenicity
Borrelia recurrentis, carteri, etc. (Louse group).		Man.*	Man (blood).	Cyclical development in louse. Bite punc- ture contaminated by crushed louse. Re- lapsing fever.
Borrelia duttoni, novyi. (Tick group.)	Tick (species of Ornith- odorus or Argas).	Man.*	Man (blood).	Excretions of tick con- taminating tick-bite, Tick fevers. Relap- sing fever.
Treponema pallidum and pertenue.	Man.	Not required.	Man.	T. pallidum. Usually venereal. Syphilis. T. pertenue. Flies or contact. Yaws.
Leptospira ictero- haemorrhagiae.	Man (rat).	Not required.	Rat.	Common infection of rats. Presentin blood. Excreted in urine. Ingestion. Weil's disease.
Leptospira morsus- muris.	Man (rat).	Not required.	Rat.	Man inoculated by bite of infected rat Rat bite fever.
Rickettsia prowazeki.	Man.	Louse (P. hu- manus, var. cor- poris.)	Man (blood).	Cyclical development in louse. Bite punc- ture inoculated by louse faeces. Typhus fever.
Rickettsia rickettsi. (Dermacentroxenus rickettsi.)	Man (goats, rodents, etc.).		l .	Excretions of tick contaminating tick bite. Rocky Mountain spotted fever.
Bartonella bacilliformis.	Man.	Species of Phlebotomus.	Man (blood).	Transmitted by species of Phlebotomus. Inoculative. Carrion's disease.
Filtrable virus of yellow fever.	Aedes mos- quitoes.	Man.	Man (monkeys).	Cyclical development in mosquito. Inoc- ulative.
Filtrable virus of den- gue fever.	Aedes mosquitoes.	Man.	Man.	Cyclical development in mosquito. Inoculative.
Filtrable virus of pap- pataci fever.	Phlebo- tomus.	Man.	Fly.	Cylical development in fly. Inoculative.

NOTE.—Although these diseases are caused by parasites which are now classified with the bacteria rather than the protozoa, or by filtrable viruses, they have been retained in this table because they resemble protozoal diseases, particularly in the mode of transmission and in the life cycle of the causative agents.

<sup>\*</sup> Some authorities regard man as the definitive host of Borrelia.

#### HELMINTHIC DISEASES-TREMATORES

Parasite	Defin. host	Intermediate host	Important reservoir of virus	Transmission and pathogenicity
Clonorchis sinensis.	Man (cats, dogs, hogs, rats and mice).	Ist, snail (species of Parafossar- ulus and By- thinia) and 2d, fish.	Man.	Eating raw fish. Ingestive. Human liver fluke disease.
Opisthorchis felineus.	Man (cats dogs and hogs).	ist, mollusc. (Dreissena polymorpha). 2d, fish.	Cats and dogs.	Man probably infected by eating raw fish. Ingestive. Liver fluke disease.
Fasciolopsis buski.	Man (pig).	Species of Plan- orbis and Seg- mentina.	Hog.	Ingestion of cercariae encysted on water plants. Intestinal Distomiasis.
Heterophyes heterophyes.	Man (dogs and cats).	Ist, Pironella conica and probably other snails. 2d, fish.	Dogs and cats.	Ingestion of raw fish. Intestinal Distomiasis.
Paragonimus ringeri.	Man (dogs, cats and hogs).	Ist, snail (species of Melania). 2d, crab.	Cats, dogs and hogs.	Eating raw crabs containing cercariae. Ingestive. Lung fluke disease.
Schistosoma haema- tobium.	Man.	Snail (species of Bulinus (Isidora?) and possibly others).		Bathing or drinking water containing cer- cariae. Penetrative. Vesical bilharziasis.
Schistosoma mansoni.	Man.	Snail (species of Planorbis and possibly others.)		Bathing or drinking water containing cer- cariae. Penetrative. Rectal bilharziasis.
Schistosoma japonicum.	Man.	Snail (species of Katayama, On- comelania and Schistoso- morpha.)	Man (faeces) (domesticated animals).	Bathing or drinking water containing cer- cariae. Penetrative. Katayama disease.

NOTE.—Rare trematodes of man include: (1) Fasciola hepatica; (2) Dicrocoelium dendriticum; (3) Metagonimus yokogawai; (4) Echinostoma ilocanum; (5) Watsonius watsoni; (6) Gastrodiscus hominis; (7) Troglotrema salmincola.

### HELMINTHIC DISEASES (Continued). CESTODES

Parasite	Defin, host	Intermediate host	Important reservoir of virus	Transmission and pathogenicity
Diphyllobothrium latum.	Man.	Ist, Cyclops stre- nuus and Diap- tomus gracilis. 2d, fish.	Man (facces), dog, cat and bear.	Eating raw fish containing plerocercoid larvac. Broad Russian tape-worm disease.
Diphyllobothrium mansoni.	Dogs, cats, other car- nivores.	Ist, cyclops. 2nd, frog, snake, rarely man.	Frogs.	Ingestion of cyclops?. Application of raw frogs to ulcers. Cysts in subcutaneous tissues, often in orbit.
Hymenolepis nana.	Man. Rat.	Not required.	Children (facces). Rat.	Man intermediate and definitive host. In- gestive. Dwarf tape- worm disease.
Hymenolepis diminuta.	Man and rat.	Rat fleas and other arthropods.	Rat.	Cases occur rarely in children. Probably ingestion of rat flea.
Taenia saginata.	Man.	Cattle.	Man (faeces).	Eating insufficiently cooked beef contain- ing cysticerci. In- gestive. Beef tape- worm disease.
Taenia solium.	Man.	Hog. (Rarely man.)	Man (faeces).	Eating insufficiently cooked pork containing cysticerci. Ingestive. Pork tapeworm disease.
Echinococcus granu- losus.	Dog and other carnivores.	Man, cattle, sheep and hogs.	Dog (faeces).	Dogs infected at abattoir. Hydatid disease.

Note.—Rare cestodes of man include: (1) Dipylidium caninum; (2) Species of Davainea; (3) Drepanidotaenia lanceolata; (4) Multiceps multiceps (cocnurus stage); (5) Sparganum proliferum; (6) Diplogonoporus grandis. (See text.)

# HELMINTHIC DISEASES (Continued). NEMATODES

. Parasite	Defin. host	Intermediate host	Important reservoir of virus	Transmission and pathogenicity	
Wuchereria bancrofti (Filaria bancrofti).	Man.	Mosquito (vari- ous species).	Man infected (blood).	Indirect in mosquito. Mature larva penetrates skin. Elephantiasis, etc.	
Loa loa (F. loa).	Man.	Species of Chrysops (mangrove flies).	Man infected (blood).	Indirect (cyclical) in Chrysops. Probably inoculative. Ocular filariasis, etc.	
Mansonella ozzardi.	Man.	Culicoides furens.	Man.	Indirect in Culicoides. Inoculative. Non-pathogenic.	
Acanthocheilonema perstans (F. perstans).	Man.	Species of Culi- coides.	Man infected (blood).	Transmission by Culi- coides. Non-patho- genic.	
Dracunculus medinensis.	Man.	Species of cyclops.	Man infected (subcutane-ous tissue).	Larvae enter cyclops. Infected cyclops in drinking water. In- gestive. Guinea worm infection.	
Onchocerca volvulus (Filaria volvulus). (O. caecutiens.)	Man.	Species of Simu- lium and Eu- simulium.	Man. (Lymph spaces of skin.)	Simulium flies. In- oculative. Subcuta- neous tumors and lymphangitis. Blind- ness.	
Strongyloides ster- coralis.	Man.	Not required.	Man (faeces).	Parasitic filariform larva penetrates skin. May cause severe diarrhoea.	
Necator americanus and Ancylostoma duodenale.	Man.	Not required.	Man (faeces).	Strongyloid larvae penetrate skin. Ancylostomiasis.	
Trichinella spiralis.	Man (rat and hog).	Hog (man and rat).	Hog (muscle).	Encysted larva in raw or insufficiently cooked pork. Inges- tive. Trichinosis.	

Note.—Ascaris, Trichuris and Enterobius (Oxyuris) do not require intermediate hosts. With Ascaris and Trichuris, larva gradually develops in egg passed in faeces. Infection by ingestion of embryo-containing eggs. Embryo-containing eggs contaminate fingers from crushing female Enterobius in perineal region.

### ARTHROPODAN DISEASES

Parasite	Life history	Disease and manifestations and remarks
Linguatula serrata (Linguatulidae).	Adult in nasal cavity of dogs, etc. Eggs in nasal mucus contaminate grass. Rabbits, cattle infected. Larvae in liver, lungs.	Porocephaliasis. Man may harbor larva, rarely adult. Larvae usually in lungs or liver and do not seem to cause symptoms.
Armillifer armillatus (Linguatulidae).	Adults in lungs of snakes. Eggs contaminate water or food. Larvae in liver, lungs, etc., of lions, monkeys, rats, man, etc.	Porocephaliasis. Larvae in liver, lungs and other organs.
Demodex folliculorum (Demodicidae).	All stages passed within hair follicles or sebaceous glands, especially about nose. Adult may wander.	Demodectic acariasis. Causes a resistant itch in dogs. In man, may invade eyelids or Mcibomian glands, but appears to be harmless.
Sarcoptes scabiei (Sarcoptidae).	Female lives in burrow of skin giving off eggs which hatch into larvae.	Scabies. Burrows show as black- ish lines, especially between fin- gers, flexor surfaces of arms and penis. Itching worse at night.
Pediculoides ventricosus (Pediculoididae).	Female lives on wheat-straw worm or grain moth. Larval mites de- velop inside mother.	Orain itch. The mites leave wheat straw or grain and attack harvesters or those sleeping on straw mattresses. Attack upper trunk, neck and arms. Erythematous or vesicular eruption with constitutional symptoms.
Trombicula irritans (Trombidiidae).	Adults live in fields or woods. The larval mite lives on grass-hoppers or small rodents.	Autumnal erythema. The larval mites known as harvest mite, red bug or jigger attack man causing a severe itch.
Dermanyssus gallinae (Parasitidae).	Mites live in chicken houses and feed on fowls.	Poultryman's itch. The mites attack man producing eczematous dermatitis on backs of hands and forearms.
Liponyssus bacoti.	Tropical rat mite. May bite man.	Conveys endemic typhus to man in southern U. S. The rickettsias are transmitted to the eggs.
Glyciphagus domesticus (Tyroglyphidae).	Mites found in flour and sugar.	Grocer's itch.
Dermacentor andersoni (Ixodidae).	Adults live on cattle, sheep, etc. May bite man.	Tick paralysis. The bite of this tick or species of Ixodes may produce paralysis in sheep. An ascending type of paralysis due to tick bites has been several times noted in man, chiefly in children.
Pediculus humanus, var. corporis (Insecta, Pediculidae).	Adults live on clothing or hair and feed on man.	Pediculosis—Phthiriasis. Produce skin irritation with (later) pig- mentation (Vagabondismus).

(torcel).

Disease and manifestations

and remarks

Site of penetration shows as black

spot with whitish induration surrounding it. Apt to form ulcers.

Larva causes a swelling with black opening. May invade eye.

## ARTHROPODAN DISEASES (Continued)

Tunga penetrans (Der- Impregnated female penetrates skin Sandflea or chigoe infestation.

Dermatobia hominis Eggs of fly become attached to A cutaneous myiasis. Larva at (Oestridae: Bot Flies). mosquitoes or ticks. These latter first club-shaped (ver macaque).

bite man and larva penetrates skin. Later worm-shaped

Life history

Parasite

(Tungidae).

matophilus penetrans) of man or animals.

		opening. may mvade eye.
	Larvae of these or other flies burrow under skin.	Creeping eruption. Larva migrans. The burrows make zigzag lines on face or soles of feet. Causes itching.
Cochleomyia americana, Chrysomyia bezziana (Muscidae). Oestrus ovis (Oestridae).	Fly deposits eggs in nostrils, ears or open wounds. Larvae wander to nasal sinuses. May invade aural canal.	Screw-worminfection. Nasal myiasis. Larvae in their wandering destroy tissues of nasal cavities, or of ear, and may cause death. May infest wounds or vagina. C. americana causes American nasal myiasis, C. bezziana causes Indian nasal myiasis and O. ovis causes African nasal myiasis.
Cordylobia anthropophaga (Ochromyia anthropophaga) (Muscidae).	An African fly which deposits eggs on clothes or sand. Larvae (African skin maggot) attack children or small animals.	Larvae bore under skin, causing boil-like lesions with central open- ing. Larvae mature in two weeks.
Auchmeromyia luteola (Muscidae).	An African fly; deposits eggs on floors of native huts. Larvae bite man.	Congo floor maggot. The bite is not painful.
Calliphora vomitoria, Lucilia caesar, L. ser- ricata (Muscidae). Phormia regina, Cochleomyia macell- aria and C. americana.	Blow and green-bottle flies, de- positing eggs on tainted meats. Larvae may be found in faeces.	Larvae may be cause of intestinal myiasis; may be found in nasal cavities or in skin wounds caus- ing serious symptoms, or in car.
Musca domestica (Muscidae).	Eggs presumably deposited near genitalia or ear.	Larvae have been found in male urethra and in the ear and in the intestinal tract.
Sarcophaga carnaria, Wohlfartia vigil (Sar- cophagidae).	Viviparous. Larvae are deposited on decaying flesh (wounds, orifices of body).	Larvae gain access to wounds, nasal cavities, etc., at times causing death. Commonly found in intestinal myiases. May occur in vagina.
Anthomyia pluvialis (Anthomyidae).	Larvae deposited in body orifices.	Occasionally reported as found in ear.
Fannia canicularis (Anthomyidae).	Eggs deposited near external genitalia and larva penetrates urethra.	Symptoms of urinary irritation or obstruction. Has been found in gastrointestinal tract.

# CHAPTER XVIII

# THE PROTOZOA

Cı	TION OF PROTOZOA			
Class	Order	Genus	Species	
Sarcodina (Rhizopoda). Move usually by means	Gymna-	Endamoeba	E. histolytica E. coli E. gingivalis	
of protoplasmic projections called pseudopodia.	moebida	Endolimax Iodamoeba Dientamoeba	E. nana I. bütschlii D. fragilis (T. gambiense	
		Trypanosoma	T. rhodesiense T. cruzi (L. donovani	
Flagellata (Mastigophora) Move by means of undulating membranes or flag-	Monozoa e	Leishmania	L. infantum L. braziliensis L. tropica	
ella.		Trichomonas	T. hominis T. vaginalis	
	Dist	Chilomastix Embadomonas Enteromonas	E. hominis	
Infusoria (Ciliata)	Diplozoa	Giardia	G. lamblia	
These have contractile vacuoles and numerous fine cilia which are shorter than flagella and have a sweeping stroke.  Sporozoa		Salantidium	B. coli N. faba	
These have no motor organs. They live para-	Coccidi- ida	Eimeria	E. stiedae	
sitically in the cells or		(Isospora	I. hominis	
tissues of other animals. Reproduction by spores.	Haemos- poridia Sarco-	Plasmodium	P. vivax P. malariae P. falciparum P. ovale	
	sporidia	Sarcocystis 404	S. tenella	

# GENERAL CONSIDERATION OF PROTOZOA

By the term protozoa we understand a branch of animals in which the individual is composed of a single cell morphologically and functionally complete; it is not one of a number of cells going to make up a complex individual and dependent upon such a combination as is the case with the metazoa (there is no differentiation into tissues in protozoa).

Cytoplasm.—Protozoal cells are made up of protoplasm which is divided into nucleus and cytoplasm. The cytoplasm is at times separated into an external, hyaline portion, the ectoplasm or ectosarc, and an internal granular portion, the endoplasm or endosarc. The functions of the ectosarc are protective, locomotor, excretory and sensory; those of the endosarc trophic and reproductive. Protozoa may be holozoic (animal-like) or holophytic (plant-like), saprophytic (fungus-like), or parasitic (living at the expense of some other animal or plant).

Nucleus.—The nucleus is characterized by concentration of the so-called chromatin substance of the cell. This chromatin, however, is usually combined with achromatin. The usually accepted test for chromatin, the staining affinity for basic aniline dyes, is now known to be unsatisfactory as other substances than chromatin may stain even more intensely. When chromatin is scattered through the cytoplasm, as extranuclear aggregations, such chromatin granules are called chromidia. There are cells where the chromidia take the place of the nucleus and from which a nucleus may be formed. Chromidia may arise from nuclei and nuclei from chromidia. The nucleus is made up of a network of linin, an achromatic reticulum in which is contained the nuclear sap or karvolvmph. As a rule an achromatic nuclear membrane, continuous with the reticulum, separates the nucleus from the cytoplasm. In addition we have a substance which is achromatic (plastin) and which is the imbedding substance for chromatin grains. These plastin chromatin combinations are called karyosomes. The nucleoli are probably pure plastin. Plastin is to be regarded as a secretion or modification of chromatin made to serve as a matrix for the chromatin. Chromatin may be concentrated so that the nuclear space looks like a vesicle with a central chromatin mass (vesicular nucleus) or numerous chromatin grains may be scattered through the nuclear space (granular nucleus). The centrosome, which presides over cell division, is usually located just outside the nucleus. In some protozoa, however, the centrosome is within the nucleus and is often seen inside a karyosome, being then called a centriole. The centrosome may also function over kinetic activities (flagellar motion) and is then termed blepharoplast.

When appearing as a small granule at the base of the flagellar apparatus, it is called the basal granule. When there are extensions from it to the nucleus we have rhizoplasts.

Certain protozoa, as trypanosomes, show a differentiation of nuclei, the larger trophonucleus governing the functions of general metabolism and the smaller kinetonucleus directing the motor activities. Infusoria have a larger macronucleus which contains vegetative chromatin and a smaller micronucleus which contains reserve reproductive chromatin.

Reproduction.—Reproduction of protozoa may be by fission, when the nucleus and cytoplasm divide into two by simple division.

When the nuclei divide into a number of daughter nuclei, this nuclear division being followed by multiple division of the cytoplasm, we have sporulation.

Instead of fission there may be sexual reproduction or conjugation (zygosis). Here the nuclei of the separate sexual individuals (gametes) are termed pronuclei and the product of their fusion a synkaryon.

Where a single cell has division of its nucleus with subsequent fusion of these daughter nuclei to form a synkaryon the process is termed autogamy.

If two similar cells conjugate, the term is isogamy; if dissimilar, as the macrogametes and microgametes of malaria, anisogamy.

The process of sexual union is termed syngamy and is of two kinds, (1) when the two gametes fuse completely (copulation) and (2) when they remain separate and only exchange nuclear material (conjugation).

Organelles.—Protozoa have structures, termed organelles, that are concerned in the functions of locomotion, metabolism, etc. To those having to do with movement, the names pseudopodia, flagella, cilia and myonemes (contractile fibrils which give support to the body cells of certain protozoa) may be applied, and to those having to do with metabolism, food vacuoles and contractile vacuoles.

#### SARCODINA

The class Sarcodina is divided into the subclasses Amoebea and Proteomyxa, only the former concerning us medically. The Amoebea are creeping forms with branched root-like pseudopodia. The pseudopodia serve the double purpose of nutrition and locomotion. These protoplasmic extensions may be quite broad or very narrow—the lobose and the reticulose. The pseudopods do not anastomose. Sarcodina showing anastomosis of fine branching pseudopods are considered as Proteomyxa.

Some species of Sarcodina have hard shell-like coverings which are secreted in or on the ectosarc. These skeletons have openings through which the pseudopods project. The pseudopodia may be made up only of ectoplasm, or both ectoplasm and endoplasm may take part. Amoeboid movement always starts in the ectoplasm. In addition to the nucleus, which the so-called chromatin-staining method of Romanowsky brings out as reddish areas, or iron haematoxylin as black, we frequently observe aggregations of chromatin-staining material in the cytoplasm. These cytoplasmic chromatoid bodies (chromidial bodies) are of importance in differentiating the encysted pathogenic amoeba from the non-pathogenic species. Food vacuoles and contractile vacuoles are present in many species of Sarcodina.

#### Amoebiasis

By amoebiasis is understood infection with the pathogenic amoeba, Endamoeba histolytica. The organism establishes itself in the large intestine, penetrating into the tissues of the intestinal wall and causing a characteristic type of chronic ulcerative colitis, associated (in some of the cases) with the clinical symptoms of amoebic dysentery. From the intestinal lesions the organisms frequently metastasize through the portal

vein to the liver, causing an hepatitis or abscess, or rarely to other tissues (lungs, brain, etc.). Four other distinct species of amoebae are known to establish themselves in the gastrointestinal tract of man, but it is generally believed that they are harmless saprophytes growing in the intestinal contents, and they have not been shown to invade the tissues or to cause disease symptoms.

Endamocha histolytica (Entamocha histolytica).—First observed by Lambl (1859) and described by Lösch (1875) in the stools and ulcers of cases of chronic dysentery, this organism has been the object of intensive study, especially since the important observations of Councilman and Lafleur (1891) on the pathological lesions of the disease, and of Schaudinn (1903) on the cytology of the parasite.

The organism as it occurs in the tissues, and in the faeces in acute dysentery, is usually 20 to 30 $\mu$  in diameter. Unstained it has a homogeneous, greyish, translucent,

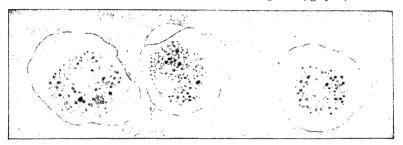


Fig. 82.—Endamoeba histolytica. The same living individual drawn at brief intervals while moving. (From Doftein after Hartmann.)

finely granular endosarc, and a clear, hyaline, highly refractile ectosarc which is best seen in the pseudopods. The single, delicate, vesicular nucleus is barely distinguishable or usually quite invisible. The organism is actively phagocytic and, unlike the saprophytic amoebae, frequently contains red cells and occasionally tissue fragments, but never bacteria or food particles or (except old or degenerated organisms) vacuoles. In a perfectly fresh preparation on a warm stage it shows active motility, moving rapidly across the field, usually in a definite direction, in a manner which Dobell and O'Connor have compared to that "of a slug travelling at express speed." The protoplasm appears to flow across the field without much preliminary extrusion of pseudopods. The other amoebae as a rule are much more sluggish and do not show such directional movement. E. coli, however, may show similar motility. This activity subsides after a few minutes, but for some time the organism continues at intervals to throw out abruptly large, blunt, blade-like pscudopodia of clear, hyaline ectoplasm. They are very susceptible to chilling or overheating. They quickly round up then and die, and can no longer be identified with certainty. The organisms in this free-living stage are known as trophozoites, or less appropriately as "vegetative" forms. They are found, often in large numbers, in the dysenteric stools, especially in the blood-tinged mucus; in scrapings from the base of the ulcers (proctoscope); in the tissues around the ulcers; in the walls of the liver

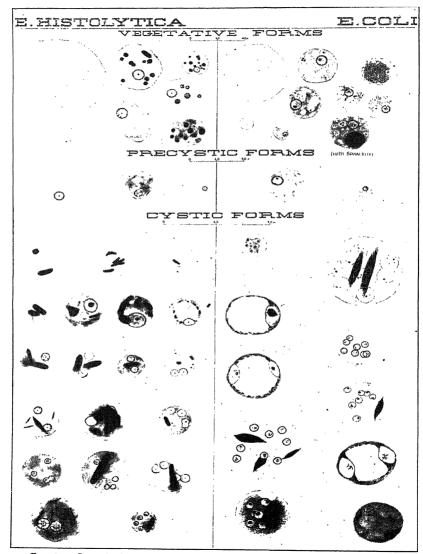


Fig. 83.—Intestinal protozoan parasites. U.S. Naval Medical School chart.

abscesses; and (inconstantly) in the pus or discharge from these abscesses. They multiply in the tissues by fission.

Some of the organisms leave the tissues and (if not too quickly expelled) undergo encystment in the intestinal contents. At this stage the organisms cease to ingest red cells or tissue fragments. They undergo two successive divisions, forming small "precystic" forms one half to one third the diameter of the trophozoites (at one time termed "E. minuta"). These never contain inclusions, but show glycogen when stained with iodine. They are sluggishly motile and otherwise resemble trophozoites. They soon round up and secrete a wall around themselves to form a cyst. The single nucleus then undergoes two successive divisions, resulting in the production in the mature cyst of four small nuclei (rarely eight), about  $2\mu$  in diameter, but identical in structure with those of the trophozoites. The nuclei are difficult to see in fresh preparations, but easily seen if a little Lugol's solution is added. They tend to be scattered at different poles of the

#### Table

Differential characteristics of *immature* cysts of E. histolytica and E. coli, as illustrated in Fig. 8<sub>3</sub>.

E. histolytica

E. coli

(r nucleus). Nucleus squeezed slightly. Chromatoidal chips more numerous and larger than in *E. coli*. Glycogen vacuole smaller than in *E. coli*. Relatively common.

(2 nuclei). Glycogen vacuole is smaller; nuclei not squeezed. Chromatoidal material in atypical masses around the glycogen vacuole. These bi-nucleate cysts with glycogen vacuoles are rarely seen.

(3 nuclei). Typical chromatoidal bodies numerous, with rounded ends. One nucleus larger than the other two or in division. Glycogen vacuole very small or invisible. Rarely seen.

(4 nuclei). Adult form. Chromatoidal bars with rounded ends. Glycogen vacuole has usually disappeared.

(1 nucleus). Rim of cytoplasm between glycogen vacuole and cyst wall narrower and chromatoidal chips smaller and fewer in number than in *E. histolytica*. Rarely seen.

(2 nuclei). Nuclei are squeezed by glycogen vacuole. Chromatoidal material scanty. Relatively common.

(4 nuclei). The nuclei are larger than those observed in an 8-nucleated cyst, and one or two of the four in process of dividing, are larger in size than the others. Chromatoids in bars with splintered end, in spicules or filiform strands. Rarely

(8 nuclei). Adult form. Chromatoidal material in filaments, wedges or chips. Glycogen vacuole has usually disappeared.

Note.—In the cyst of *E. histolytica*, chromatoid matter occurs in three forms: (1) Primary chromatoid bodies (present in cysts taken directly from faecal material soon after the stool is passed. (2) Latent chromatoid matter (microscopically invisible, present in cysts left in the faecal material for ten to eighteen hours). (3) Secondary chromatoid bodies (develop from latent chromatoid matter when cysts are placed in water). These are always in the shape of rods with rounded ends, or bars. Therefore, it is frequently of diagnostic value to place a portion of the faeces in water for 6 to 8 hours or overnight, and then look for the more characteristic secondary chromatoid bodies, particularly in identifying the small race of *E. histolytica* cysts. (Hakansson).

#### THE PROTOZOA

cyst. The cysts are usually 10 to  $15\mu$  in diameter. They may vary from 5 to  $20\mu$  in different strains, although the cysts of a given strain are fairly uniform in size. In the cytoplasm of the cysts there is often one or more chromidial bodies, coarse, globular or cigar shaped, highly refractile, greenish structures which do not stain with iodine, but stain deeply like chromatin in fixed films. In the younger cysts there may be also one or more diffuse glycogen masses which stain deeply with iodine. They disappear as the cyst matures. Cysts are found only in solid or semisolid faeces, not in the dysenteric stools. They are never formed in the tissues or in the liver abscesses.

In films stained with iron-haematoxylin the details of the nuclear structure can be made out. This is the decisive point in differentiating the various species. In both the trophozoites and the cysts there is a narrow, beaded, peripheral ring of chromatin, and a minute central dot (karyosome) without any granules in the intervening space. (See Table and Fig. 83 and 84 for comparison with other species.)

Pathogenesis.—Human infection occurs only as a result of the ingestion of cysts. In the large intestine the cysts undergo ("metacystic") development, each finally giving rise to 4 small trophozoites, which attach themselves to the epithelium, especially in the crypts. They penetrate into the tissues of the mucosa and often quickly into the submucosa, partly by their own active movements and partly by means of a lytic substance which they secrete. Here they give rise to small areas of gelatinous necrosis (abscesses) which rupture into the lumen of the intestine and produce ulcers subsequent course depends upon the balance between the destructive powers of the organism and the reparative powers of the host. In most cases the latter suffice to restrict the lesions to small and even microscopic dimensions, and the infection is symptomless. It is doubtful, however, if the defensive forces are adequate (without treatment) to eliminate the infection entirely, once it is established in the tissues. In other cases the organisms penetrate into the submucosa, and extend laterally in this layer, undermining the mucosa and leading to the formation of large ulcers. Secondary bacterial infection from the intestine then occurs. The portions of the bowel wall between the ulcers are commonly not inflamed. The muscular coat is relatively resistant, but in the severest cases it may be penetrated as well as the serosa, giving rise either to perforation and general peritonitis or to the formation of adhesions to neighboring structures. In severe chronic cases there are extensive adhesions, marked scarring of the intestinal wall, in some places with thinning and dilatation, in others with thickening of the wall and narrowing of the lumen, and occasionally the formation of tumor-like masses of granulation tissue. A striking feature of all the lesions (intestinal and hepatic) is the absence of leukocytic infiltration unless secondary bacterial infection occurs. Any part of the large intestine (including the appendix) may be involved, but practically never the small intestine. Sites of predilection are the caecum, the flexures, and the rectum (see Meleney, 1035).

Clinically, the severity of the symptoms varies as greatly as does the extent of the ulceration. The incubation period is variable and generally long, the onset is usually insidious and the course protracted, marked by recurring periods of active dysentery alternating with periods of remission during which there may be troublesome constipation. The disease may last 30 or 40 years. There may be merely a mild diarrhoea, or the dysentery may be severe, with 12 or more bowel movements daily, accompanied by marked colicky pain and tenesmus. Rarely the onset may be fulminant, and death may occur within a week. As a rule the onset is less abrupt and the symptoms less acute than in bacillary dysentery. The disease may be symptomless, or be associated

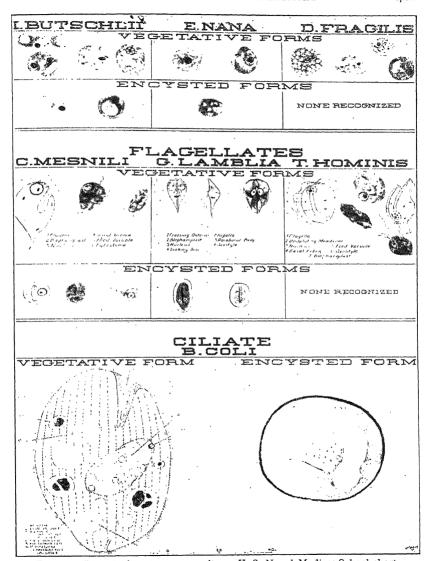


Fig. 84.—Intestinal protozoan parasites. U. S. Naval Medical School chart.

with vague abdominal pains, digestive discomforts, or other complaints which do not suggest intestinal disease. These mild or symptomless cases have been shown to outnumber greatly the cases with clinical dysentery. They constitute the carriers or "cyst-passers." It is generally believed that the organisms cannot maintain themselves in the intestinal contents and that the presence of cysts is proof that the tissues have been invaded, although the lesions produced may be insignificant. In such individuals (if untreated) the carrier state is probably permanent. A carrier may develop clinical symptoms at any time if his resistance is lowered, but most of them never do so. The reasons for such marked variations in the reaction of different individuals to the infection is not understood. Some evidence that different strains of amoebae vary in virulence has been obtained by Meleney and Frye (1933).

Distribution.—Amoebiasis is wide spread in the tropics and subtropical regions. It seems to be especially prevalent in Indo-China, China, and the Philippines as well as in parts of India. It is also very common in Egypt and in northern and central Africa. In South America, especially Brazil, it is common, and also in the West Indies and Central America. It is an important disease in the Southern States of the United States, as well as in Italy and Southern Europe. It is much more common than has been generally believed in the Northern States and in northern Europe, as shown by the considerable number of carriers in these regions, although clinical dysentery is relatively rare. Many studies have been carried out to determine the frequency of symptomless carriers. The figures vary considerably, but indicate that from 3% to 5% of the population in the classes examined are infected in England, France, and the Northern United States. Thus Stiles found 4.1% of 13,043 Americans infected, but Andrews and Paulson found only 0.2% (of adults) infected. In some limited groups in institutions or camps, much higher figures (from 10% to 19%) have been found.

*Epidemiology.*—The disease is acquired solely by the ingestion of cysts. The trophozoites are destroyed by the stomach juice. Therefore the disease is not spread by the patient with acute dysentery, but only by the faeces of convalescents and carriers.

The cysts are killed quickly by desiccation or by heat, but may remain infective for at least two weeks in moist faeces or in water (maximum 153 days, Boeck). The commonest modes of transmission are: (1) Contamination of food or water by the lingers of infected food handlers—by far the most important in the cities of civilized countries under ordinary living conditions. This explains the usually sporadic occurrence of the disease. (2) Contamination of food or water by flies which have fed on infected faeces. Cysts have been found in large numbers in the faeces of these insects (Musca domestica, Fannia canicularis, Lucilia, Calliphora), and have been shown to remain viable at least 48 hours. (3) Eating uncooked fruits and vegetables which have been fertilized with infected human faeces—a common custom in the Orient. (4) Contamination of a water supply by infected faeces. This is most likely to occur in country districts, or army camps where drinking water is obtained, unfiltered, from open wells or streams which are exposed to contamination with surface water from polluted soil. Such water should always be boiled, as chlorination does not kill the cysts.

Water so contaminated may give rise to explosive epidemics. A remarkable example of such an epidemic occurred in Chicago in 1933, in which over 800 cases developed among the employees and guests of two large hotels. This was traced to an error in

plumbing; a direct communication had been inserted between a sewer pipe and the intake pipe supplying drinking water, through which it was possible for a reverse flow from the sewer pipe to take place. Cysts were found in water drawn from the pipe in the vicinity of this communication. Many of the food handlers had been infected, and doubtless contributed to the spread of the infection.

The incubation period of dysenteric symptoms was determined with relative precision in a large number of cases in this epidemic. It varied from 7 days to more than three months, but was four weeks or less in nearly two thirds of the cases and three weeks or less in half the cases (McCoy and Hardy, 1936). Presumably the incubation period may be shortened if the infecting dose is massive. For the same reason, perhaps, the clinical course was unusually acute and severe in many of these cases.

Human experiments.—The infectivity of the cysts and their causal relation to amoebic dysentery was proved by Walker and Sellards (1913). They carried out feeding experiments on volunteers from a Philippine prison. They first proved that amoebae cultivated (by the methods then available) from water, vegetables and dysenteric stools did not colonize in the intestine or produce disease. They then fed 20 individuals with material containing  $E.\ coli.$  Of these 17 became parasitized after from 1 to 11 days, but no one developed symptoms of dysentery. Walker then fed 20 individuals with faeces containing  $E.\ histolytica$  cysts. Of these 18 became infected (1 only after 3 feedings) after an average interval of 9 days. Of the 18, only four developed dysentery, after intervals of 20 to 95 days. He also fed 4 individuals with material from dysenteric stools or pus from liver abscesses containing trophozoites, but not cysts, with entirely negative results.

Animal inoculation.—Schaudinn, Craig and Wenyon and many others have shown that kittens or puppies can be infected by the administration by mouth or by rectal injection of material containing pathogenic amoebae. Sellards and Baetjer were able to infect all their kittens by injecting the material directly into the caecum, and propagated a strain of amoebae through a series of animals for several months. The intracaecal inoculations yielded positive results in cases of human amoebiasis in whom the clinical manifestations were obscure, and the amoebae in the discharges so few and atypical as to make such an examination unsatisfactory. Darling suggested utilizing injections into kittens of material containing amoebae as a means of differentiating E. histolytica from E. coli (which is non-pathogenic for these animals). The dysentery produced in kittens differs from the disease in man in that the inflammation is more acute and more generalized, and that cyst formation has not been observed. Monkeys (Macacus rhesus) may also be infected, and sometimes contract the disease spontaneously.

Laboratory Diagnosis.—The gross and microscopic appearance of the stools in acute cases is of considerable value, particularly in differentiating amoebic from bacillary dysentery. In amoebic dysentery the stools are usually fluid, relatively copious, and contain faecal material, varying amounts of fresh and altered blood which give them a dark brownish or reddish color, and much blood-streaked or brownish mucus. They are often fetid. This contrasts with the scanty, watery, non-faecal passages containing masses of whitish mucus flecked with bright red blood in the

bacillary type. Microscopically the amoebic stools show mucus, and numerous red cells, often clumped and degenerated, but very few pus cells or phagocytic cells which are numerous in the bacillary type (see p. 125). The cells which are present mostly show cytolysis, and consist of scanty ragged cytoplasm surrounding pycnotic nuclei. Charcot-Leyden crystals are highly suggestive but not pathognomic of amoebic dysentery.



FIG. 85.—Stool in bacillary dysentery (early stage) showing (A) macrophages containing light bodies surrounded by a halo—red blood cells and dark bodies which are probably nuclear detritus. Polymorphonuclear leukocytes are numerous and many show a ringing of the nucleus resulting from a toxic degeneration. (Army Medical Museum No. 39105.) Compare with Fig. 86.

Demonstration of trophozoites or cysts in the stools is essential for a positive diagnosis. (See Chap. XXXVI, Examination of the Faeces.) In the active stage of the disease look for trophozoites in particles of blood-tinged mucus fished from a fresh warm stool. The material may be mounted in warm physiological salt solution. We have obtained beautiful results with vital staining by tinging the suspension with 1% aqueous solution of neutral red. Examine with the low power (% inch objective, 6×

ocular), and use the high power for identification only. Magath (1935) has emphasized the great practical importance of this point. If the stool is formed, motile organisms can rarely be found in the faecal mass, but may be demonstrated in mucus adhering to it. In such cases one may give a saline purge and examine flecks of mucus contained in the first

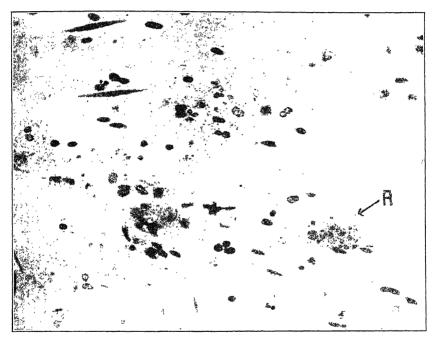


FIG. 86.—Stool in amoebic dysentery showing two amoebae one of which (A) contains red blood cells. There are a number of red blood cells throughout the smear some of which show a tendency to clump. Charcot-Leyden crystals are present. (Army Medical Museum No. 38180.)

fluid stool. Andrews (1934) and others have shown that the chance of finding protozoa is much increased by such catharsis. Oil (and barium) must be avoided.

If there are ulcers in the rectum, the organisms can usually be demonstrated easily in scrapings from the ulcers obtained through a proctoscope. They can often be obtained more simply by passing a rectal tube as deeply as possible into the rectum and examining the fleck of mucus caught in the eye of the tube. The organisms are

### THE PROTOZOA

not evenly distributed in the stool and several particles should be examined. They must be differentiated from the non-pathogenic amoebae (see Table for differences) and from free-living saprophytic amoebae, occasionally present.

The trophozoites, if they do not contain red cells, can not always be distinguished with certainty from those of E. coli, either in fresh or stained preparations. It must be remembered that the characteristic features of the trophozoites and cysts of the various species of intestinal amoebae enumerated can not be made out in every individual, but that in every faecal specimen there are atypical forms, particularly of the trophozoites, which can not be positively identified. Several individual organisms should always be inspected.

Amoebae must also be distinguished from large mononuclear phagocytic cells, such as those common in the stools in bacillary dysentery. The latter have larger, more conspicuous nuclei than E. histolytica, and often contain bacteria or vacuoles and even red blood cells. It is essential for diagnosis to demonstrate the typical motility of the parasite. This together with the presence of ingested red cells practically suffices to identify an organism as E. histolytica. If there is doubt as to the identification, or if permanent preparations are desired, films must be fixed and stained by the iron-haematoxylin method (see p. 869) and the nuclear structure studied. This procedure should supplement, not replace the examination of fresh preparations. Aside from the time required for staining, it is more difficult to detect amoebae in stained films than in fresh preparations.

During the first few days after the onset of the initial attack the organisms may be absent or sparse. At later periods they can usually be found. They may be absent or sparse in one specimen and abundant in the next, but examination of properly selected material from three suitable specimens will reveal them in nearly all cases.

The cysts should be looked for in solid or semisolid faeces. Cysts and trophozoites may be present in the same specimen. Practically, identification depends upon distinguishing the four nuclei. This is difficult and often impossible unless the preparation is mixed with dilute iodine solution, or Wenyon's stain (see p. 744). Chromidial bodies should not be mistaken for nuclei. In the case of strains which form small cysts ( $_5$  to  $_7\mu$ ) differentiation from Endolimax may be difficult and require fixed stained films. They must also be differentiated from Blastocystis (p. 438).

Cysts are unevenly distributed in faeces, and are often sparse. One should examine material from several particles, or prepare a homogeneous suspension from a considerable portion of the stool. Dobell concluded that examination of a single stool would reveal cysts in only a third of infected cases, and to exclude infection with reasonable certainty 6 examinations are required. Svensson and Linders demand 10 examinations. The concentration method is of assistance but requires great care on the part of the examiner to avoid infecting himself.

Concentration of cysts. No method is satisfactory. The following procedure (after Yorke and Adams) gives fairly good results. Rub up about 10 Gm. of faeces in a mortar with tap water and emulsify thoroughly in 500 cc. of tap water. Let settle 15 minutes in a cylinder. Remove and discard the surface scum, and siphon off the fluid, leaving the sediment and about one inch of the water column undisturbed. Put

the fluid suspension in a cylinder and let it sediment over night, or centrifugalize in 50 cc. tubes. Siphon off and discard the fluid, suspend the sediment in a little water and centrifugalize. Wash the sediment two or three times with water, and examine for cysts.

Pus from liver abscesses is characterized by its very viscid, tenacious consistence and chocolate-brown color. It is usually streaked or mixed with fresh blood in varying degrees. Rarely it is creamy and yellow. This pus gives to the sputum its "anchovy sauce" appearance in cases in which the abscess ruptures into a bronchus. Microscopically such "pus" consists largely of granular cellular detritus, red blood cells, and occasionally large phagocytic cells and a few leukocytes. Pus cells, however, are usually sparse or absent unless there is secondary bacterial infection. It may contain cholesterin, haematoidin, or Charcot-Leyden crystals. Motile amoebae may be numerous, but are usually sparse or absent in material obtained by aspiration, or in the discharge for a few days after drainage has been established. Cysts are never found.

Cultivation of E. histolytica was first accomplished by Boeck and Drbohlav (1925) on Locke-egg-scrum medium. Dobell suggested the use of Löffler's blood scrum, to which a little albumin-Locke solution and rice starch is added just before inoculation. The liver infusion agar-scrum medium of Cleveland and Collier (p. 855) has been recommended. At the National Institute of Health favorable results have been obtained with Boeck's egg slant overlaid with Ringer's or Locke's solution to which is added 250 mg. of dehydrated Löffler's blood scrum per liter. A loop full of faeces (either solid faeces containing cysts or dysenteric stool) is rubbed up in the overlying solution, and incubated 24 to 48 hours. The sediment is examined microscopically for amoebae. Growth is influenced by the contaminating bacteria, some favoring, others tending to inhibit it. By subculturing at 2 to 4 day intervals strains have been maintained for long periods and retain their infectivity for kittens and monkeys. Under favorable conditions encystment (and excystation) occurs.

Craig and St. John and a number of others have advocated the use of cultures for routine diagnostic purposes, claiming the procedure gives positive results more simply and more frequently than microscopic examination. Magath and others deny this and point out the difficulty of accurately distinguishing in cultures the trophozoites of E. histolytica from those of the four saprophytic species, which can also be cultivated (Svensson and Linders), although with greater difficulty. Free living amoebae accidentally ingested with the food may also grow in such cultures. Further experience is necessary to determine the practical value of the method.

Complement fixation.—Craig (1929) has demonstrated that the serum of infected patients may give a positive reaction with a suitably prepared antigen (from cultures). The technique is difficult, and the reliability of the results has not yet been demonstrated sufficiently to warrant use of the procedure for clinical diagnosis.

### THE NON-PATHOGENIC AMOEBAE

Endamoeba coli (Grassi, 1879) is the largest of the amoebae occurring in man (20 to  $40\mu$ ). The cytoplasm is granular and shows no clearly defined hyaline ectosarc. It may contain food vacuoles, bacteria, and starch granules, but no red cells. The nucleus is more sharply outlined than in E. histolytica, is visible in fresh preparations, and contains more chromatin. The pseudopods, which are greyish and lobose, are projected sluggishly, and the organism usually shows little or no progressive motility although in warm salt solution it may do so. The cysts are somewhat larger than those of

### THE PROTOZOA

E. histolytica and when mature contain 8 small nuclei. These are more easily seen and tend to be aggregated in the center of the cyst. Chromidial bodies are usually absent or in the form of fine splinters or threads. Larger chromatoids have splintered ends, in contrast to the smooth blunt ends of those of E. histolytica. The organism is fairly common, being reported in 15% to 25% of several groups of soldiers examined.

Endolimax nana (Wenyon and O'Connor, 1917) is the commonest of these amoebae, being reported by Kofoid in 28% and by Dobell in 33% of soldiers examined by them. It is small (6 to  $12\mu$ ) and contains a single small ( $2\mu$ ) nucleus in which the chromatin is largely clustered in a single coarse irregularly shaped karyosome, the "limax type" of nucleus. In fresh preparations it is sluggishly amoeboid, and may contain food vacuoles. The cysts are the same in size and contain 4 minute nuclei but no chromidial bodies or glycogen masses. They resemble the small strains of E. histolytica cysts.

Iodamoeba bütschlii (Provazek, 1912) (I. williamsi) is a small amoeba (9 to  $12\mu$ ) resembling small forms of  $E.\ coli$  in its sluggish motility and granular cytoplasm, which contains food particles. The single small nucleus has the chromatin largely concentrated in a central karyosome. In films stained with iron-haematoxylin they closely resemble  $E.\ nana$ . The trophozoites are much rarer than the cysts. The cysts ("iodine cysts") are the same in size, are oval or irregular, and contain a single small nucleus, a large compact mass of glycogen, but no chromidial bodies. Although it is believed to be harmless and not to invade the tissues, it is highly susceptible to emetine treatment, unlike  $E.\ coli$  and  $E.\ nana$ . Although its distribution is extensive it is relatively rare. Manson-Bahr states that it is found "in about 5% of faeces, most commonly in those who have been in the tropics, and not infrequently in association with  $E.\ histolytica$ ." Hegner and Taliaferro, however, report an incidence of 10% to 15%. It occurs in monkeys and is common in hogs.

Dientamoeba fragilis (Jepps and Dobell, 1918) is a relatively small amoeba, 3 to 12µ in diameter. It does not progress and its motility consists of the extrusion and withdrawal of clear "fingers" of ectoplasm. There are two nuclei present in most instances, but as many as 40% have been observed with but one nucleus. The arrangement of the nuclear chromatin is typically characteristic and diagnostic. The chromatin is arranged in a ring of granules, 4, 5 or 6 in number, about midway between the central point of the nucleus and the extremely fine nuclear membrane. Occasionally a very fine dot can be made out in the center of the nucleus which may be a karvosome. When stained with iron-haematoxylin, if the process of differentiation has not been carried sufficiently far, the nucleus may appear to have a large solid karyosome. amoebae are often difficult to fix and stain properly. Bouin's fixative has been recommended as giving better results than Schaudin's fixative. Cysts are not definitely known. Although this amoeba is wide-spread, it has been considered rare. However, Wenrich et al. (1935) reported finding this parasite in 4.3% of 1060 cases of University of Pennsylvania students. As the organism disintegrates rather rapidly in the faeces and is relatively difficult to stain, it is doubtless often overlooked. It has been regarded as non-pathogenic, but a few cases have been observed in which it appeared to be the cause of a dysentery, and further study of its pathogenicity is needed.

Endamoeba gingivalis is a (probably) harmless saprophyte living in the mouth, especially in pyorrhoeal pockets. It is a sluggishly motile organism to to  $25\mu$  in diameter, with sharply differentiated ectoplasm and endoplasm which contains many inclusions (food particles, etc). The nucleus is small (2.5 to  $3\mu$ ), vesicular, with a distinct nuclear membrane and a deeply staining karyosome. Cysts are probably not formed.

Caudamoeba sinensis, described by Faust as the cause of a dysentery in China, is believed by Wenyon to be a form of E. histolytica.

	Endamoeba tetragena of Viereck has been shown to be identical with E. histolytica.
	KEY TO GENERA AND SPECIES OF AMOEBAE (DOBELL AND O'CONNOR)
ı.	(a) One nucleus present in active amoeba
	(b) Two nuclei present
2.	(a) Nucleus with small spherical karyosome and peripheral layer of fine chromatin
	beadsGenus Endamoeba 3
	(b) Nucleus with large irregular eccentric karyosome and no peripheral chromatin
	granules
	(c) Nucleus with large central spherical karyosome surrounded by a layer of achro-
	matic granules Genus Iodamoeba 5
3.	(a) Ripe cyst, 4 nuclei; glycogen diffuse; large chromatoids generally present
-	E. histolytica.
	(b) Ripe cyst, 8 nuclei: glycogen in early stages only; large chromatoids occasion-
	ally present but often absent
4.	Ripe cyst, 4 nuclei; glycogen rarely present; chromatoids absentE. nana.
	Ripe cysts, I nucleus; glycogen in a dense mass; no chromatoids

### FLAGELLATA (MASTIGOPHORA)

6. Nuclei with central granular karyosomes and no peripheral chromatin. (Cysts 

In this class of protozoa the adults have flagella for the purposes of locomotion and the obtaining of food.

Some flagellates more or less resemble rhizopods in being amoeboid and in having an ectoplasm and an endoplasm. The body is frequently covered by a cuticle (periplast). Some flagellates have a definite mouth part, the cytostome, which leads to a blind oesophagus; others absorb food directly through the body wall. In addition to flagella, some flagellates possess an undulating membrane. All flagellates possess a nucleus and some have contractile vacuoles. The flagellum may arise directly from the nucleus or from a small kinetic nucleus, the blepharoplast (micronucleus or basal granule).

The most important flagellates of man are the haemoflagellates, Trypanosoma and Leishmania. In addition flagellates occur in the intestine and in the vaginal secretion.

The Trypanosomidae.—The members of this family are probably primarily insect parasites, some of which have become partially adapted to vertebrates or to plants.

There are 4 morphological types: (1) leishmania; (2) leptomonas; (3) crithidia; and (4) trypanosome (see Fig. 87). The most primitive form is the leptomonas from which the others are derived. In this type the kinetoplast from which the flagellum

## DIFFERENTIATING CHARACTERISTICS OF PARASITIC AMOEBAE (AFTER DOBELL AND O'CONNOR)

### MOTILE STAG

		MOTHE SIAGE		
	Budamoeba histolytica	Bndamoeba coli	Endolimax nana	Iodamoeba bütschlii
Size.	2030μ	20-30µ	η21-9	9-13и
Mobility.	Characteristic. Freshly removed from host displays astonishing activity. Flows astonishing activity. Plows field. Soon becomes less active pushing out a few large, blunt, blade-like pseudopodia which are perfectly hydnied are perfectly are perfectly and perfectly are perfectly and perfectly are perfectly and perfectly are perfectly and perfectly and perfectly and perfectly and perfectly are perfectly and perfectly and perfectly and perfectly and perfectly are perfectly are perfectly and perfectly are perfectly and perfectly are perfectly and perfectly are perfectly and perfectly are perfectly are perfectly and perfectly are perfectly are perfectly and perfectly are perfectly and	Freshly removed may show considerable activity, as a rule, however, extremely sluggish with little locomotory movement. Motions consist chiefly in changing of shape without evident progression. Formation of large, clear, blade, like pseudopolia rarely seen. Degenerate, motionless or dead forms frequently indistinguishable from similar forms of E. histolytica.	As a rule not very active. Has sign v progressive movement in fresh preparations. Later shows no movement save frew, blunt and thick. Soon mounts up and dies and this more or less degenerate form is the one most commonly seen in stools.	Generally but slightly motile. Movements smilar to E. coli. Greatly resembles when alive small specimens of E. coli. Quickly degenerates and dies.
Cytoplasm	Bndoplasm colorless, finely granular, and uniform in appearance. May contain red blood cells and fragments of terriar and other particles in host's faeces probably never ingested normally.	Endoplasm bulky, granular and usually contains numerous food vacuoles charged with bacteria, yeasts, vegetable debris and other particles derived from host's facees. Red blood cells not ingested. Other vacuoles, spindle-shaped, containing figuid seem. No sharp line of demarcation separates the ectoplasm from the endoplasm.	Displays few noteworthy features. Endoplasm finely gran- tures. Endoplasm finely gran- ular with numerous minute food vacuoles containing in- gested bacteria. Red blood cells not ingested.	Displays few peculiarities. Endoplasm finely granular and homogeneous in appearance. Usually contains numerous food vacuoles charged with minute bacteria. Red blood cells not ingested. Cysts of this amocha have been previously described as "Iodine cysts."
Nucleus	4-7µ.—Unstained a delicate vesicle inconspictous or invisible. Stained shows fine chromatin granules lining wall giving a finitely beaded ring appearance. Karyosome small, spherical and usually central. Chromatic part o.5µ in size.	4-7µ.—Unstained readily distinguishable. Stained shows larger beads of chromatin ling wall. Karyosome, spherical, usually eccentric and larger than that of history fica. Chromatic part Iµ in size.	1-3µ.—Stained the medeus is vesicular with a delicate membrane free from chromatin. All chromatin contained in a large, irregular, eccentrically placed karyosome. Variations in size and shape of karyosome characteristic of this species.	2-3.5µ.—Unstained readily distinguishable as a small vesicle with a distinct membrane. Stained shows wall free from chromatin as a rule. Typically echromatin almost entirely contained in a large, central, spherical karyosome. Zone between muclear wall and karyosome filled with single layer of small granulies.

# DIFFERENTIATING CHARACTERISTICS OF PARASITIC AMOEBAE (AFTER DOBELL AND O'CONNOR) (Continued)

## PRECYSTIC STAGE

AND THE PERSON NAMED IN COLUMN TWO IS NOT THE PERSON NAMED IN COLUMN TWO IS				
	Endamoeba histolytica	Endamoeba coli	Endolimax nana	Iodamoeba butschlii
Remarks	Active amoebae in lumen of gut undergo division leading to decrease in size. Therefore, these forms vary in size. Red cells and other food particle expelled. By secreting delicate, transparent wall become cysts. Nucleus structurally similar to that of adult.	ketive amoebae in lumen of Pornation same as with E.  gut undergo division leading histolytica. Often indistin- to decrease in size. Therefore, these forms vary in size. Red In fact degenerate forms of cells and other food particles either species can never be expelled. By secreting deli- differentated with certainty and lead to error in diagnosis. yests. Nucleus structurally similar to that of adult.	Unlike species of Endomocho this stage is not smaller than the adult. Differs only in not containing food inclusions. Nucleus structurally same as that of adult.	Active amoebae in lumen of Formation same as with E. Unlike species of Endamoeba Same size as adult. No food gut undergo division leading histolytica. Often indistin- this stage is not smaller than inclusions and the cytoplasm to decrease misse. Therefore, guishable from E. histolytica, the adult. Differs only inclusions. Nucleus differs from adult cells and other food particles either spectes can never be Nucleus structurally same as in containing more granules and lead to error in diagnosis. Nucleus structurally similar to that of adult.

## ENCYSTED STAGE

			No. of Contract of	
Size 7-15µ	7—15µ	15-20μ	π6	9-12μ
Shape	Round.	Round.	Usually oval.	More or less rounded.
Wall	Thin.	Thicker than E. histolytica.	Thin.	Relatively thick.
Nuclei	[ -	Typically shows four nuclei. Typically shows eight nuclei. Wery small. May show one to four. Nuclei May show one to twenty. May show one to the four nuclei. Structurally similar to adult type of nucleus. Structurally similar to adult type of nucleus.  May show one to four nuclei. Rarely Differs structurally from the targandes to a didutty per in the clear zone become that of adult type of nucleus.	Very small. May show from one to four nuclei. Rarely eight. Structurally similar to adult type of nucleus.	One relatively large nucleus. Differs structurally from the adult type in that the granules in the clear zone become massed at one pole giving an eccentric karyosome.
Chromatoids	Large chromatoids with blunt, rounded ends common.	Chromatoids Large chromatoids with blunt, Large spicular chromatoids may Absent. rounded ends common.	Absent.	Absent.
Glycogen	Glycogen Diffuse but not abundant.	Relatively abundant in the Rarely present, early stages. Scanty or absent in mature cysts.	Rarely present.	Dense glycogen mass is characteristic.

arises is near the anterior end, and there is no undulating membrane. In the crithidia the kinetoplast is near but still anterior to the nucleus. The axoneme of the flagellum passes from the kinetoplast to the convex margin of the body and thence along its surface, or on the edge of an undulating membrane, to the anterior end of the body and becomes the flagellum. The free edge of the membrane is longer than the attached margin, hence the membrane is thrown into folds. In the trypanosome form the

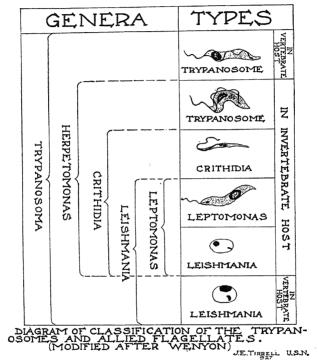


Fig. 87.—Trypanosomidae.

kinetoplast is posterior to the nucleus, and the axoneme passes along an undulating membrane from the kinetoplast to the anterior end of the body. In the leishmanial form the nucleus and the kinetoplast are contained in a small round body, and the axoneme extends from the kinetoplast to the periphery of the body. This form may be assumed by any of the preceding types.

On the basis of their morphology and life cycle Wenyon has differentiated 6 genera: (1) Leptomonas; (2) Crithidia; (3) Herpetomonas; (4) Phytomonas; (5) Leishmania; and

- (6) Trypanosoma. The members of the first three genera are intestinal parasites of

invertebrates and are transmitted by encysted forms in the faeces. The members of the fourth genus are similar to the *Leptomonas* morphologically, but require both plants and invertebrates in their life cycle. The members of the last two genera (the haemoflagellates) are communicated to man and other vertebrates by the bites of certain arthropods. The *Leishmania* occur only in the leishmania and leptomonas forms like the *Leptomonas*, from which they are differentiated by the fact that they have a vertebrate as well as an invertebrate host in which either phase may occur. The *Trypanosoma* resemble the *Herpetomonas* in that all four forms exist, but differ in that they have both vertebrate and invertebrate hosts.

### TRYPANOSOMES OF SLEEPING SICKNESS

The African trypanosomiases are due to infection with Trypanosoma gambiense or Trypanosoma rhodesiense. The former is transmitted by the bite of a tsetse fly, Glossina palpalis, and the distribution of the disease corresponds with that of these flies. The affected areas are scattered throughout central Africa, particularly along the Congo river and its tributaries. The latter is transmitted by another species of tsetse fly, Glossins morsitans, and occurs in relatively limited areas in Rhodesia, Mozambique and Tanganyika. T. brucei, the cause of a devastating disease of horses, dogs and cattle (nagana), also transmitted by G. morsitans, is believed by many to be identical with T, rhodesiense. Some authorities claim that the T. rhodesiense was derived originally from T. gambiense and modified by transmission through a different species of fly and by its introduction into a previously unaffected area. In western Nigeria another species of fly, G. tachinoides, has been found to be infected with a trypanosome which is apparently identical with T. gambiense. Brazilian trypanosomiasis (Chagas' disease) is caused by a distinct species, T. cruzi, and is transmitted by winged reduviid bugs, Triatoma megista in Brazil, Rhodnius prolixus in Venezuela, and other vectors.

### African Sleeping Sickness

African sleeping sickness begins as an irregular, remittent fever with tachycardia, which may last for months or even years, with or without intermissions. A more or less generalized lymphadenitis develops, most frequently in the posterior cervical glands. Trypanosomes may be found in these glands as well as in the blood stream. Peculiar skin rashes and local areas of oedema, particularly about the eyes and joints, are frequent. After a variable and often long period of time a meningo-encephalitis supervenes, owing to the invasion of the central nervous system by the parasite, and the characteristic symptoms of the stage of sleeping sickness appear. With the appearance of these symptoms the prognosis becomes

practically hopeless, although death may not occur for a year or more. Trypanosomes are present in the cerebrospinal fluid, and have been demonstrated in the substance of the brain.

The rhodesiense type of sleeping sickness runs a more rapid course than does the gambiense type and usually causes death within a year.

Natives of the infected regions have a greater resistance to the disease than have white men, and trypanosomes have been found in the blood in apparently healthy individuals. Such cases are important from the standpoint of epidemiology.

Trypanosoma gambiense. Morphology.—This flagellate has a thin, slender, curved, fish-shaped body, the anterior end of which tapers to a fine point while the posterior end is relatively blunt. An undulating membrane extends almost the entire length of the

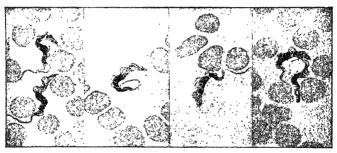


Fig. 88.—Trypanosoma gambiense (slide presented by Professor F. G. Novy). (From Todd.)

body, the margin of which is longer than the attached edge so that it is thrown into folds. In stained films two chromatin-staining areas are visible. The large trophonucleus is central. Posteriorly there is a deeply staining oval structure called the kinetoplast. From a minute granule (the blepharoplast or kinetic nucleus) in this body an axial filament or axoneme arises and extends anteriorly along the free margin of the undulating membrane and forwards into a single, long, whip-like flagellum. Scattered granules of chromatin may be visible in the anterior part of the body. In fresh preparations an active writhing or lashing motility is observed. Progression is usually in the direction of the flagellated end, but occasionally is in the opposite direction. The size of this trypanosome is variable. The normal forms found in the blood may be from  $1-3\mu$  in width. Long forms up to  $33\mu$ , which are preparing to divide, may be found. The normal short form is believed to be the only form from which development takes place in the fly. This species of trypanosome shows unusual pleomorphism in the blood of vertebrates—a useful point in differentiation.

Multiplication in vertebrates is usually by amitotic longitudinal fission, division of the kinetoplast and trophonucleus preceding that of the cytoplasm.

Culture.—Both of the human trypanosomes of Africa have been cultivated in various modifications of the N.N.N. medium. Rat blood or human blood is more favorable than that of the rabbit. Growth is difficult to secure, however, and subcultures are apt to die out quickly. The trypanosome of the Brazilian disease is more readily cultivated. The development of the organism in cultures resembles that in the invertebrate host.

Transmission.—T. gambiense is transmitted by the bite of a tsetse fly, Glossina palpalis. Mechanical transmission by a fly, whose proboscis has been contaminated with trypanosome blood within a few hours, may occur and has been demonstrated experimentally. Generally, however, the infection is derived from the bite of a fly in which there has occurred a developmental cycle of the trypanosome. This requires about 18 days or more after an infected feed, and during this period the bite is harmless. Once infective, however, the fly remains so the rest of its life—up to 185 days. Only a small proportion of the flies that feed on infected blood become infective (approximately 2 to 6%). Infection is not transmitted to the pupa.

The cyclic development of T. gambiense, T. rhodesiense, and T. brucei in the fly is similar and is peculiar to these types. After the fly ingests the infected blood, the parasites accumulate and multiply in the middle and posterior portion of the gut. Between the 8th and 18th day long slender forms appear, pass forward into the proventriculus, and from there into the salivary glands and ducts. Here further development takes place into crithidial forms, which attach themselves to the gland cells. From these are developed the infective "metacylic" trypanosomes, similar to the normal short type seen in vertebrate blood, which pass down the salivary ducts and through the channel in the hypopharynx, from which they enter the bite wound. Only these "metacyclic" trypanosomes are infective for vertebrates; the forms in the digestive tract of the fly are harmless.

Epidemiology.—The disease is transmitted only by the bite of the tsetse fly. Although infected human beings are probably the most important source of infection for the vector, there is evidence of a reservoir of infection in the wild game of the region, particularly in various species of antelopes. T. rhodesiense has been found in the blood of apparently healthy antelopes, and it is probable that these animals may serve as a reservoir for both types of the disease. Domestic animals may also be a factor in the spread of the infection.

Of the preventive measures the most efficacious have been those directed toward eradication of the flies. The tsetse fly haunts the underbrush within a few yards of some stream, where the females deposit their larvae. Deep shade is essential for their development, and it has been found that clearing out the underbrush for a distance of 20 to 40 feet on either side of the water courses is an effective measure. Since these

flies are not prolific, eradication has been possible in isolated areas by means of traps. Segregation of human cases from flies and treatment of such cases to rid the blood of trypanosomes are important, but the chronicity of the disease and the fact that apparently healthy natives may carry the parasites in the blood lessen the feasibility of these measures. The flies tend to follow moving objects, and with the opening up of new roads in Africa, there is great danger of introducing trypanosomiasis into hitherto unaffected areas. In some regions guards are posted to inspect all travellers and vehicles for flies before allowing them to proceed.

Pathogenicity.—Most of the laboratory and domestic animals as well as many wild animals are susceptible, although in none of them is it possible to reproduce the stage of sleeping sickness as it occurs in man. Some species of monkeys appear to be immune. Different strains of T. gambiense vary in virulence and generally produce only a mild infection in animals, while T. rhodesiense is much more virulent.

Laboratory diagnosis.—Typanosomes may be demonstrated directly in thick films from the blood stained with Leishman or Giemsa stain, or, preferably, in a fresh preparation, but since the parasites are usually sparse, better results are obtained by centrifugalizing the blood to concentrate them. Broden uses citrated blood (9 cc. of blood to 1 cc. of 6% sodium citrate solution), centrifugalizing it twice at 1000 and at 1500 revolutions for 10 minutes. The supernatant fluid and leukocyte cream are removed and centrifugalized at a higher speed for 20 minutes. Smears made from the sediment are then examined for trypanosomes.

Smears from the gland juice may show the trypanosomes, when they cannot be found in the blood. The material is obtained by puncturing an enlarged gland. The syringe and needle used must be dry, since water distorts the parasites.

In the stage of sleeping sickness trypanosomes can often be demonstrated in the cerebrospinal fluid. There is an increase in the globulin content and in the cell count, in which the type of cell depends upon the chronicity of the infection.

If the trypanosomes cannot be found in smears, a rat or guinea pig may be inoculated with the material. Feebly virulent strains may produce only a mild infection, but examination of the blood at intervals will usually reveal the trypanosomes.

When the organism cannot be demonstrated the following tests of the patient's serum have been used for diagnosis. (1) The formol-gel test, described in the section on Leishmaniasis, is said to be positive in outspoken cases (Morrison and Dye). (2) The so-called autoagglutination test has been used. In a fresh blood preparation the red cells become aggregated into large clumps which may be visible with the naked eye. They are not rouleaux. The reaction is not specific for trypanosomiasis, however, and is not of much diagnostic value. (3) Trypanolysis may be demonstrated by mixing unheated (fresh) serum with a suspension of trypanosomes and incubating for an hour.

The reaction is said to be positive in a majority of the cases. (4) The adhesion phenomenon. A mixture of inactivated serum, leukocytes (or any particulate matter) and trypanosomes is set up as described on p. 147. In a positive reaction the cells or particles adhere to the trypanosomes. The reaction is specific for the different species, and has been used in epidemiological studies in wild animals.

The practical value of the latter procedures is restricted by the technical difficulties in securing suitable suspensions of trypanosomes.

Trypanosoma rhodesiense.—This trypanosome is indistinguishable morphologically from T. gambiense in human blood, but may be differentiated by passage through laboratory animals, preferably rats. In the animal blood some of the T. rhodesiense have the nucleus displaced toward the posterior end, so that it is adjacent to the kinetoplast or actually posterior to it. The proportion of such forms is usually small, about 5%, varying with different strains. The same phenomenon is exhibited by T. brucei.

This trypanosome is more virulent for animals than *T. gambiense*, and the disease in man runs a more acute course and is less responsive to arsenical therapy.

Transmission is through another species of tsetse fly, G. morsilans, but the developmental cycle in the fly takes place in the same manner.

The relationship between this species and  $T.\ brucci$  is still unsettled. If they were identical one might expect rhodesiense trypanosomiasis to be more common than it is, since the species of fly that transmits both occurs over a large area, and animal infections with  $T.\ brucci$  are frequent. Furthermore, inoculation of volunteers with the latter has failed to produce the disease in man. Yorke and his collaborators suggest that this apparent lack of pathogenicity for man may be due to individual immunity, since many normal sera exert a lytic action on  $T.\ rhodesiense$  to which  $T.\ gambiense$  is resistant. If such a natural immunity exists, some of the epidemiological facts are more easily understood.

### The Trypanosome of Chagas' Disease

Trypanosoma cruzi (Schizotrypanum cruzi) Cruz and Chagas, 1909.— This trypanosome causes a disease of varied symptomatology occurring in Brazil, Venezuela, Argentina and neighboring regions. The principal vector is a reduviid bug, Triatoma megista. Chagas created a new genus (Schizotrypanum) for the organism because of the intracellular proliferation of the leishmanial forms; but the morphological characters conform to those of the trypanosomes, and most authorities prefer to retain it in the genus Trypanosoma.

The disease occurs in an acute form in infants and children. It is characterized by fever, general glandular enlargement, and enlargement of the spleen, liver and thyroid. Puffiness of the face with conjunctivitis is a common and characteristic early feature. Trypanosomes are present in the blood in the acute stage. Occasionally they invade

the central nervous system and produce a fatal meningo-encephalitis. Cases which recover from the acute stage may develop chronic manifestations. In adults a chronic type of the disease has been described in which trypanosomes are not demonstrable in the blood but occur in foci in various tissues and organs in the body. The clinical picture depends upon the areas involved. A myxoedematous syndrome is relatively frequent, and cardiac and nervous types are recognized. Invasion of the adrenals, producing the picture of Addison's disease, has been described. Some strains of the trypanosome seem to have very little virulence, since they have been found in the blood of apparently healthy individuals. The lack of any distinctive characteristics of the disease, and the prevalence of endemic goitre and other diseases among the natives make the diagnosis difficult. These facts have led some recent investigators to question the existence of a chronic form of the disease.

Morphology.—This trypanosome occurs in the blood in two forms, one rather broad and the other relatively narrow. The latter are young forms according to Brumpt.



Fig. 89.—Trypanosoma cruzi in blood of child with acute type of Brazilian trypanosomiasis. (MacNeal from Doflein after Chagas.)

They average about 20µ in length and are somewhat curved. The nucleus is central and the kinetoplast is oval and large. Dividing forms are never seen in the blood. The trypanosomes invade the tissue cells, particularly those of the voluntary muscles and heart, the central nervous system, thyroid, adrenals, and bone marrow. Within the cell the trypanosome loses the flagellum and undulating membrane and assumes a rounded leishmanial form which rapidly undergoes repeated binary fission. This proliferation converts the cell into a cyst-like structure. Within 4 or 5

days these forms develop a flagellum and become transformed into a crithidial form, and finally into a trypanosome of the blood type. These are liberated by the rupture of the cell and eventually regain the blood stream.

Cultures can be obtained without difficulty in blood broth or on N.N.N. medium. Subcultures are more difficult to secure, and the organism loses its virulence on prolonged cultivation.

Transmission.—The principal vector of the disease is the Triatoma megista in which a developmental cycle takes place. According to Brumpt the ingested trypanosomes develop in the mid gut into crithidial forms which multiply rapidly. Within 10 to 20 days the infective metacyclic trypanosomes appear and are excreted in the faeces. This cycle occurs in the larval and nymphal as well as in the adult stage of the bug. Once infective the bug remains so for the duration of its life, and it has been shown experimentally that practically all of the bugs fed on material containing the trypanosomes become infective. Human infection is caused by contamination of the bite wound, or other abrasion, with faecal matter from an infected bug, or by its ingestion. The frequent occurrence of inflammation of the conjunctiva has suggested that this

may also be a portal of entry for the trypanosomes. Most of the investigators agree that the infection is not transmitted by the bite.

Epidemiology.—The Triatoma megista is found in great numbers in the houses and sheds of the poorer classes where it lives in the cracks in the walls, emerging at night to feed on man and on domestic animals. It is called barbiero (or barber) by the natives because it has a predilection for biting on the face. The adults can fly short distances in search of food. Infected bugs have been found far from human habitation, and it is believed that wild animals, particularly some of the armadillos, are reservoir hosts of this trypanosome. Infected bugs have been found inhabiting the burrows of the armadillo, and it is probable that when the burrow is abandoned the bugs migrate to the nearest source of food. Naturally infected opossums and, recently, bats have been found; and dogs and cats may harbor the infection. In addition to the Triatoma megista, a number of other species of the Reduviidae, several species of bed bugs, and also mites and ticks may be infected and are potential vectors according to Brumpt. Infected reduviid bugs have been found in California and in Utah, but no cases of human trypanosomiasis have been reported in the United States. Preventive measures should be directed toward ridding the houses of bugs.

Laboratory diagnosis.—In acute cases in children T. cruzi may be found in thick films of the blood. They are usually scanty, however, and may be demonstrated more frequently by centrifugalizing the blood to concentrate them. All of the ordinary laboratory animals can be infected, and guinea pig inoculation with blood may demonstrate the trypanosomes when they are too sparse to be found in films. After about 2 weeks they are numerous and easily found in the blood of the animal, and the proliferating leishmanial forms may be seen in the organs. Brumpt advocates the "xeno-diagnostic" method. Laboratory-bred third stage larvae of Triatoma megista are fed with the suspected blood, and after about 2 weeks the intestinal tracts are examined for parasites.

These trypanosomes are seldom found in the lymphatic glands. They are present in the spinal fluid in the acute cases with meningo-encephalitis. Biopsy of an affected muscle has been used for diagnosis.

Positive results have been obtained by these methods in only about a third of the cases. Lacorte in 1927 devised a complement fixation test with which he claimed to obtain positive results in from 80 to 90% of suspected cases. His antigen was prepared from the heart of a heavily infected puppy. Of these cases only 17% gave a positive Wassermann reaction and these showed syphilitic lesions. A control series of normal individuals was negative. These results have been confirmed by others using extracts of the heart and spleen of infected animals as the antigen.

### Trypanosomes of Animals

Of the more important pathogenic trypanosomes of animals may be mentioned:

Trypanosoma brucei.—This trypanosome causes a fatal disease in horses and one from which few cattle recover. It is called "nagana" or the fly disease, because it is transmitted by the tsetse fly, Glossina morsitans. All mammals except man and possibly the goat seem susceptible. The disease is characterized by fever, oedematous areas about the neck, abdomen and extremities, progressive anaemia and emaciation. It is an important disease of domesticated animals in many parts of Africa.

Trypanosoma evansi.—This is the cause of a very fatal disease of horses in India and the Orient known as "surra." It also affects camels and even cattle. It is thought to be transmitted by biting flies (Stomoxys). The symptoms are fever, emaciation, oedematous areas and great muscular weakness.

Trypanosoma equinum.—This trypanosome causes a fatal disease in horses in South America. There is paralysis of the hind quarters of the horse which gives the disease the name "mal de caderas."

Trypanosoma equiperdum.—This trypanosome causes a disease of horses in many parts of the world. It is known as "dourine" and is transmitted by coitus. The genital organs show marked oedema which is followed by anaemia and paralysis.

Trypanosoma dimorphon.—This trypanosome causes a disease of horses in Gambia. It is also found in horses and cattle in other parts of Africa. The parasite shows marked variation in morphology.

Trypanosoma lewisi.—Rats in many parts of the world show this infection which is rarely fatal to them. It is transmitted by the rat flea by a process of regurgitation. It can also be transmitted by the rat louse.

There are many species of trypanosomes in birds, frogs, fish, etc.

### Leishmaniasis

Members of the genus Leishmania are responsible for generalized infections such as kala-azar (Leishmania donovani) and infantile kala-azar (L. infantum) and for at least 2 types of cutaneous infection—Oriental sore (L. tropica) and the South American muco-cutaneous disease, espundia (L. braziliensis). These organisms are practically indistinguishable morphologically, and it has not been proved conclusively that they belong to separate species either by serological tests or by their pathogenicity for animals. There are, however, epidemiological, pathological, and clinical distinctions between the visceral and the cutaneous forms of leishmaniasis which warrant the consideration of these flagellates as separate types.

Kala-azar occurs in widely distributed areas in India, China, Southern Russia, Central Africa, and the Mediterranean littoral. The disease is characterized by a chronic course, intermittent fever, and marked gradual enlargement of the spleen and to a lesser extent the liver. There is a progressive cachexia and an anaemia with marked leukopenia and occasionally an extreme granulocytopenia. It has been suggested that the anaemia may be myelophthisic, the haematopoietic tissues being crowded out by the phagocytic cells which are packed with parasites. The L.

donovani are present within the endothelial cells of various tissues, particularly the spleen, liver, bone marrow and lymphatic glands; and also in small numbers, in the monocytes and polymorphonuclear leukocytes of the circulating blood.

Kala-azar occurring along the shores of the Mediterranean differs in that it affects chiefly infants and young children, but the clinical picture is similar, except in so far as the reaction of infants to infection differs from that of adults. In these areas a similar disease exists in dogs due to a *Lcishmania* (*L. caninum*), but the relationship of the two diseases is still controversial. It is generally believed, however, that infantile kala-azar is identical with the adult type. It has been suggested that the Mediterranean basin may have been the original focus of kala-azar, and that sufficient natural immunity has been developed to protect adults but not children, who, as in old malarial districts, bear the brunt of the disease.

Cutaneous leishmaniasis (oriental sore, Delhi boil, etc.) is found in other parts of India, N. Africa, Asia Minor, Syria, Persia, Arabia and countries bordering on the Mediterranean. Although this distribution corresponds roughly to that of kala-azar, the particular districts affected are usually quite distinct. The ulcers are generally located on an exposed part of the body and may be single or multiple. They are exceedingly chronic but heal eventually after many months and produce a lasting immunity. The parasites are present in the local lesion. In the South American leishmaniasis (espundia) the cutaneous lesions are followed by deep eroding ulcers of the mucous membranes with involvement of the neighboring lymphatic glands.

The relationship of oriental sore to kala-azar is still obscure. In some cases of oriental sore evidence of associated general infection has been obtained. Leishmania from cutaneous lesions have been shown to produce generalized infection in animals, and the organism from kala-azar may produce cutaneous lesions. There is also some evidence suggesting that cutaneous infection protects to some extent against subsequent generalized leishmaniasis. Manson has suggested that their relationship may be analogous to that of vaccinia and variola. Noting that oriental sore is particularly common in camel-using countries, he assumed that repeated passage of the virus through camels had biologically modified its pathogenicity for man.

Morphology.—These parasites belong to the family Trypanosomidae and occur in the leishmanial form in man and in the leptomonas form in the bodies of various insects. The Leishmania (Leishman-Donovan bodies) are round or oval bodies averaging 2 to 5µ in diameter. The nucleus is relatively large and peripherally placed; the kinetoplast is smaller, rod-shaped or oval, and set at a tangent to the nucleus. A short, slender filament, the axoneme, is sometimes seen extending from the blepharoplast to the periphery. In a Romanowsky stain the cytoplasm is faintly blue, the nucleus appears as a mass of bright red granules, and the kinetoplast is deep reddish purple. One or more vacuoles are common.

The Leishmania multiply by binary fission within the cells of the host, which may contain as many as 200 parasites in a single cell. Eventually the affected cell becomes destroyed by this proliferation and disrupts, setting free the Leishmania which are then taken up by other endothelial cells or by the leukocytes and monocytes of the blood.

Cultures may be obtained readily in citrated blood or in a moist tube of N.N.N. medium. Growth occurs in the water of condensation. Incubation must be at a temperature of 20 to 24°C. Bacterial contamination inhibits their growth. On cultivation they develop into leptomonad forms similar to those found in insects. After about 48 hours the *Lcishmania* increase in size and elongate until they reach a size of from 14



Fig. 90. -Leishmania tropica. Smear from granulation tissue of Delhi boil or oriental sore. (MacNeal from Doflein after J. H. Wright.)

to  $20\mu$  in length by about  $2\mu$  in width. The kinetoplast is situated at the blunt anterior end, and from it a long flagellum arises. This is about the length of the body of the organism, and there is no undulating membrane. Multiplication is by longitudinal fission, and aggregations of the parasites in the shape of rosettes with the flagella toward the center may be seen. Cultures may be kept for a long time by making transfers every 2 or 3 weeks, but their virulence is apt to be lost. Wenyon and Nicolle have preserved strains in this way for over 15 years.

Transmission.—The precise mode of transmission has not been established with certainty and may vary in the different types of leishmaniasis. Generally, infection results from the bite of some insect in which a cyclic development of the parasite has occurred. In cutaneous infections, contamination of an abrasion of the skin by the virus directly, or by mechanical transfer by any recently contaminated insect, suffices. In kala-azar the Leishmania have been demonstrated in the secretions of the nose and throat, and it is possible that infection may be acquired at times by droplet infection. Shortt has shown that Chinese hamsters may contract the disease by direct contact

with a sick animal without an insect intermediary. The virus has been reported in the urine and faeces, but it is unlikely that the disease is spread in this way.

It is generally believed that the vector of Indian kala-azar is a species of sand-fly, *Phlebotomus argentipes*, which infests the regions in which the disease is endemic. When these flies are fed on infected blood, a cyclic development occurs, and great numbers of leptomonad forms are present in the mid-gut by the 4th day. In some of the flies these forms may be found in the pharynx and biting mouth parts after from 8 to 12 days. It has been demonstrated that the *Leptomonas* are ejected by the fly when feeding by allowing it to bite through a rabbit skin membrane into a sterile fluid in which they may then be demonstrated. That these parasites are virulent is indicated by the fact that typical oriental sores have been produced by rubbing the crushed sand-flies into the abraded skin, yet kala-azar has not been produced by allowing infected flies to bite human beings.

In other regions different species of sand-flies occur. P. perniciosus and P. papatasi infest the Mediterranean littoral, and instances of natural infection have been found in the latter species. It must be remembered, however, that many arthropods harbor morphologically similar species of flagellates which are not pathogenic for man. In Brazil and Paraguay the disease is apparently spread by jungle insects. Species of tabanid flies have been suspected. There is considerable evidence that Phlebotomus intermedius and possibly P. lutzi are vectors.

The possibility of transmission by other biting insects has been investigated. In some of these (bed-bugs, mosquitoes) development of the Leishmania into leptomonad forms has been shown to occur, yet they apparently do not play an important rôle in the transmission of kala-azar (Patton, Wenyon and others). Experiments with lice have been negative. Donovan suggested a Triatoma. In fleas Patton was unable to find any development, and the ingested Leishmania disintegrated and disappeared from the intestinal tract within 8 hours. It has been claimed that their development in insects is influenced by the temperature, and that this fact explains the seasonal incidence of oriental sore. Since epidemiological studies indicate that isolating an infected area for a distance of only 300 yards suffices to prevent the spread of the disease, the vector must be one with only a very limited flight. This fact would seem to eliminate mosquitoes and house flies, and lends support to the belief that sand-flies are the most important, if not the only, vector.

Reservoir hosts.—In the Mediterranean area generalized leishmaniasis in dogs is common, and it has been assumed that they constituted the reservoir of infection. Dog fleas are common, but the experiments of Wenyon and others indicate that they are not concerned in the transmission of the disease. The sand-fly (P. perniciosus), however, may be a vector since the Leishmania undergo a cyclic development within them, and they are known to bite both dogs and man. In certain regions in India where kala-azar is endemic, however, canine leishmaniasis does not occur. Furthermore, in parts of Persia where kala-azar is unknown, dogs are frequently infected. Isolated

cases of natural infection have been found in wild animals such as the Chinese hamster, and cutaneous lesions occur frequently in some domestic animals; rarely in wild animals.

Pathogenicity for animals.—Dogs, cats, monkeys, rats and mice may be infected by intraperitoneal or intrahepatic injection. The Chinese hamster is more susceptible. Infection does not develop with any regularity, and they may appear well even when parasites are present in profusion in the internal organs. In animals, as in man, a long and variable time may elapse between inoculation and the development of generalized lesions. Smith (1935) reports the development of the disease in a hamster 18 months after being bitten by an infected sand-fly. This observation is of great importance, and the long incubation period may explain in part the reported failure of previous workers to transmit kala-azar experimentally by the bites of infected insects. Three other similar instances of experimental infection of hamsters have been reported.

Laboratory diagnosis. Cutaneous leishmaniasis can be diagnosed with certainty only by demonstrating the Leishman-Donovan bodies in the local lesion. The skin at the edge of the lesion is cleansed and sterilized. A small puncture is made with a knife or needle, and material is aspirated with a sterile pipette. Cultures may be made and films prepared, stained by Leishman's or Giemsa's method, and examined directly. This method of obtaining the material is much more satisfactory than scraping the ulcer, and it is usually possible to avoid bacterial contamination. In espundia the parasites may also be demonstrated in the regional lymph glands either by puncture or by examination of an excised gland.

In kala-azar there is regularly a marked leukopenia with an increase in the monocytes of the blood. Associated with these there is a diminution in the coagulability of the blood and a lessened resistance to bacterial infection.

Demonstration of the parasites in the blood by stained films is possible in from 20 to 80% or more of the cases. They are practically always sparse, and a long and careful search of several slides may be necessary to find them. They are less often present in the blood in afebrile periods, and usually disappear during active antimony treatment. Various methods are used to concentrate the leukocytes within which the organisms are to be found. Shortt makes the films on slides, starting in the usual way and then abruptly lifting the pusher slide, forming a thick edge of blood in which the leukocytes are relatively numerous. Young and van Sant advocate centrifugalizing venous blood (obtained in a modified Locke's solution) and preparing films from the buffy coat. Wenyon believes that the Leishmania can be demonstrated more frequently by culturing the sediment from citrated blood on moist tubes of N.N.N. medium. The water of condensation is examined for flagellated forms after 10 days, but the cultures are not considered negative unless they

remain sterile for at least 20 days. Growth occurs earlier when the inoculation is heavy.

The Leishman-Donovan bodies occur in greater number in the spleen, and can be demonstrated easily in material obtained by splenic puncture. This procedure is not without danger, however, since the lessened coagulability of the blood increases the risk of a fatal haemorrhage. (It must not be undertaken until the possibility of leukaemia has been excluded.) If the liver is enlarged, puncture of this organ is preferred since it is attended with less danger, although the parasites are not so numerous as in the spleen. Movements of the diaphragm should be limited by a firm abdominal binder, and the patient is cautioned not to move or breathe while the puncture is being made. It is desirable to connect the needle to the syringe with a piece of rubber tubing. The outfit must be perfectly dry, since any trace of water will distort the organisms. The material left in the needle after the puncture is examined by cultures and stained films.

Parasites are usually numerous in the bone marrow also, and material for examination can be obtained from the sternum (or tibia) with less danger to the patient. The marrow is exposed by perforating the thin outer layer of compact bone with a small trephine or by puncture with a large needle. This procedure is discussed in detail on page 316. The parasites have been demonstrated in excised lymph glands and also in gland juice obtained by puncture.

When the *Leishmania* cannot be demonstrated the diagnosis may be confirmed by the following serological tests.

Formol-gel or aldehyde reaction.—Add a few drops of commercial formalin to I cc. of the suspected serum. If the quantity of blood is limited a drop of serum may be placed on a slide which is then inverted over a watch glass containing a few drops of formalin. In a few minutes the serum will solidify and become opaque. The reaction is positive in a majority of the cases of kala-azar. In the early stages it may be negative, and the strength of the reaction diminishes progressively during convalescence. It is usually negative in cutaneous leishmaniasis. Partial solidification may occur in some cases of tuberculosis, leprosy, syphilis and heavy malarial infections, but the serum does not usually become opaque.

Antimony test.—Add 2 drops of the serum to 2 cc. of a 1% solution of urea stibamine or other pentavalent antimony compound. Within 15 minutes a heavy flocculent precipitate occurs in positive cases. The test may be made more sensitive by layering the serum over the antimony solution. The strength of the reaction can be estimated by the amount of the precipitate. The results of this test parallel closely those of the formol-gel reaction according to Napier.

The mechanism of these reactions is not understood. Nattan-Larrier and others (1934) have shown that two factors are involved in the formolgel reaction. The substance causing gelification is removed from the serum by dialysis, while that producing opacity is retained. These investigators suggest that the two tests be combined by adding immediately to the antimony test ½ cc. of formalin. A heavy, bulky precipitate is formed. They claim that the combined test is more sensitive and more reliable than either alone.

Globulin precipitation test (Ray's test).—Mix one part of serum with two parts of distilled water. A turbidity and later a flocculent precipitate develops in positive cases. If water is poured on the surface of the serum, a ring effect will be produced. This reaction is due to a marked increase in the globulin (chiefly euglobulin) content of the serum with a corresponding reduction in the amount of albumin. The reaction is not specific for kala-azar, however, but occurs in other conditions, particularly malaria. Meleney and Wu have reported very high globulin values in Schistosoma japonicum infections also.

Sensitization tests.—Intradermal injections of alkalinized extracts of the Leishmania are said to give positive reactions in a majority of the cases. The diagnostic value of these reactions, and of complement fixation tests, is disputed.

Animal inoculation is not useful as a diagnostic procedure, since it is difficult to infect the ordinary laboratory animals with small quantities of material.

### INTESTINAL FLAGELLATES

These parasites are classified according to the number of flagella, and the presence or absence of an undulating membrane and of a blepharoplast. They are adapted to a life in a fluid medium, and appear in the stools when the contents become fluid or semifluid. In normal stools encysted flagellates alone are found. They require only the single host, man, and it is doubtful whether the human species are found in other animals. They can be cultivated on media suitable for amoebac.

They can best be detected in fresh preparations, in which their active motility makes them conspicuous objects. In a dying condition they may show some "pseudo-amoeboid" movements. To distinguish the flagella it is best to mount them in Gram's iodine solution. For this purpose we take a clean slide and make a vaseline line across it about one inch from the end. A drop of iodine solution is placed on the slide about ½ inch from the vaseline line, and a suitable portion of the faeces to be examined is emulsified in it. The edge of a square cover glass is then applied to the vaseline line and allowed to drop on the preparation. By pressure suitable thickness of the fluid can be obtained. There is no current motion. It is desirable to use dark field illumination as in this way the flagella are distinctly brought out. The India ink method is also

applicable. Films may be stained with iron haematoxylin or by Giemsa's method after fixation in methyl alcohol or Schaudinn's solution.

In addition to the species described below several others have been reported (*Cercomonas*, *Bodo*, etc.) as occurring rarely in human faeces, but it is probable that they are accidental contaminations and of no practical importance.

Trichomonas hominis (T. intestinalis) is a very common parasite in diarrhoeal stools, but as to its pathogenicity there is much doubt. It is pear shaped and about 9 by  $14\mu$ . There are three to five, usually four flagella, projecting anteriorly, while another one forms the border of the undulating membrane and projects posteriorly. An axostyle passes from the anterior to the posterior end. A cytostome is present near the nucleus at the anterior end. Cysts have not been found. They move with an irregular circular or spinning motion.

Trichomonas vaginalis is larger, 12 to 25 micra long, and differs in that the fourth flagellum does not project beyond the undulating membrane. It occurs frequently in the vagina, where it sometimes causes an acute vaginitis with profuse leucorrhoeal discharge.

Chilomastix mesnili (Wenyon, 1910) is a common flagellate which differs from the trichomonas in not having an undulating membrane or an axostyle. It is about 6 by 15 (to  $20\mu$  in size. The three anteriorly projecting flagella are long and slender. There is a long very prominent slit-like cytostome within which is a single flagellum. The posterior end is very much attenuated and tail-like. It moves in a slow, deliberate manner with slow rotation of its body. Cysts are about  $8\mu$  in length, ovoid, with a small projection at the narrower end, and contain one nucleus. Like the preceding it occurs in the large intestine, especially in the caecum.

Giardia lamblia (Lambl, 1859) is the commonest flagellate. It is pear shaped, about 10 by  $15\mu$  in size. There is a deep depression on the ventral surface at the blunt anterior end which enables the organism to attach itself to the summit of an epithelial cell. Around the depression are three pair of flagella which are in constant motion. Another pair of flagella project from either side of the blunt little tail-like projection. When stained they show a pair of chromatin-staining areas at the anterior end. There are two axostyles but no undulating membrane. When in motion they have a slow, tumbling sort of progression. The cysts are oval, 8 by  $14\mu$ , and show two axostyles and four small nuclei.

These parasites live in the upper part of the small intestine in enormous numbers. They have frequently been reported as the cause of a debilitating diarrhoea. The parasite has been reported in about 6% of groups of soldiers examined by Woodcock and Penfold in England and by Kofoid in the United States. Claims that they may cause inflammation of the gall bladder and bile ducts are not well founded. Similar if not identical parasites occur in mice, rats and other animals, but it is not certain whether man may be infected from these animals.

Embadomonas intestinalis (Wenyon and O'Connor, 1917) is about  $6\mu$  long. It is actively motile, and pear shaped. Two flagella arise from blepharoplasts on the nuclear membrane of the single nucleus which is near the blunt end of the parasite. The cysts are about  $5\mu$  long and pointed at one end.

Enteromonas hominis (Fonseca 1915) is a minute, actively motile, pear shaped flagellate, with the posterior end pointed. Three flagella project anteriorly and one posteriorly. The small cysts resemble fungus spores. Both of these species are rare and of no practical significance.

### THE PROTOZOA

The pathogenicity of these flagellates is still in dispute. Dobell holds that there is insufficient evidence for regarding any of them as pathogenic. As grounds for this view he cites the facts: (1) that there is no good reason for considering intestinal flagellates as more common in cases of diarrhoea than in well persons; (2) that parasites of the alimentary canal which do not attack the tissues of the host, as is the case with intestinal flagellates, are not harmful; and (3) that no method of treatment has been discovered which will remove such an infestation. Most authorities now believe that Giardia (and perhaps Trichomonas) may at least aggravate a colitis due to some other primary cause, and that in rare instances they may be primarily pathogenic. Once established, infection with these flagellates is extremely persistent.

Blastocystis hominis is an organism regarded as a mould, which may be mistaken for an encysted flagellate or even an amoebic cyst. It has a large central vacuole with a narrow refractile rim which contains one or more nuclei. When stained by Giemsa's stain the central part is very faintly colored, whereas the rim is deep blue. With iron-haematoxylin the center may stain very deeply, and the rim a much lighter grey or blue.

### Infusoria (Ciliata)

The Infusoria are the most highly developed of the Protozoa.

The bodies of Infusoria are oval and may be free or attached to a stalk-like contractile pedicle, as with *Vorticella*, or they may be sessile. The cilia, which are characteristic, may be markedly developed around the cytostome (mouth) and serve the purpose of directing food into the interior, while others act as locomotor organs. The body is enveloped by a cuticle which may have only one opening or slit, to serve as mouth; or it may have a second one, a cytopyge or anus. Usually the faecal matter is ejected through a pore which may be visible only when in use. They usually have a large nucleus and a small one. Infusoria tend to encyst when conditions are unfavorable (as when water dries up in a pond). When the cilia are evenly distributed over the entire body of the ciliates we have the order Holotricha; when ciliated all over, but with more prominent cilia surrounding the peristome, we call the order Heterotricha. It is to this order that the Infusoria of man belong.

Balantidium coli.—This is the only ciliate of importance in man. It is a common parasite of hogs. It is from 60 to  $100\mu$  long by 50 to  $70\mu$  broad, and has a peristome at its anterior end which becomes narrow as it passes backward. It has an anus. The ectosarc and the endosarc are distinctly marked. The cuticle is longitudinally striated.

The parasites may penetrate into the mucosa and submucosa of the intestine, carrying bacteria with them and causing abscesses which rupture into the intestine. They thus cause a chronic dysentery which may have a fatal termination. They may produce a pernicious type of anaemia. The infection is spread by cysts, round structures about  $50\mu$  in diameter. It is almost impossible to escape noticing the actively moving bodies if a faecal examination is made.

Another ciliate, the B. minutum, 25  $\times$  15 $\mu$ , has also been reported for man.

Nyctotherus faba has a kidney-shaped body and is about 25 by  $15\mu$ . It has a large contractile vacuole at the posterior end. It has a large nucleus in the center with a small fusiform micronucleus lying close to it. It has been reported only once for man,

### Sporozoa

This class of Protozoa is characterized by its method of reproduction, by sporulation. Reproduction may be asexual (by schizogony) or sexual, by the formation of sporozoites in cysts. There are no locomotor organs. All species are parasitic within the cells, tissues or body cavities.

### CLASSIFICATION (MODIFIED FROM HEGNER)

Subclass **Telosporidia**.—Spore-formation occurs only after growth is complete and terminates the life of the individual. Parasites intracellular during part of the life cycle.

Order Gregarinida.—Gametes of equal size. Usually no schizogony. Parasites in digestive tract or body cavities of worms and arthropods, rarely or never in the higher vertebrates.

Order Coccidia.—Gametes unequal. Both sexual and asexual generations occur in the same host. Zygotes form a cyst which does not increase in size. Usually parasitic within epithelial cells of the gastrointestinal tract.

Order Haemosporidia.—Gametes unequal. Sexual and asexual generations in unrelated hosts (arthropod, vertebrate). Resistant spores usually absent. Parasitic within blood cells. (Includes malaria.)

Subclass **Neosporidia.**—Spore formation occurs during life of the parasite. Usually multinucleate in adult stage.

Order Myxosporidia.—Adult a large multinucleate plasmodium. Spores large, usually with two polar capsules. (Economically very important because it includes *Nosema bombycis*, the cause of pebrine, a disease destructive to silk worms.)

Order Microsporidia.—Spores small, usually with one polar capsule.

Order Sarcosporidia.—Spores in sac-like tubules in muscle cells of vertebrates. This order contains the only parasites among the Neosporidia which have been reported for man. (Haplosporidia are now classed with the fungi.)

### Coccidia

The parasites belonging to this order are found within the epithelial cells of the intestine and the organs connected with it, especially the bile passages. They grow within the cells, which they gradually consume, and finally multiply asexually (schizogony), forming numerous minute falciform merozoites. These escape from the disintegrated cell and invade fresh epithelial cells in which the cycle is repeated. Finally certain of the parasites develop into sexual forms (gametocytes). The male (microgametocyte, 20 to  $30\mu$  long) divides into a large number of minute sperm-like bodies provided with two flagella (microgametes), one of which penetrates into and fertilizes the female macrogametocyte. Fertilization is extracellular, probably in the lumen of the gastrointestinal tract. The fertilized macrogamete then develops an exceedingly resistant membrane about itself and is known as an oöcyst. The oöcyst is passed in the faeces and constitutes the infective stage of the parasite. Its contents divide into two or four secondary cysts (sporocysts) within which a varying number of falciform sporozoites

develop. When ingested these are liberated, penetrate the epithelial cells and start the asexual stage of development. No intermediate host is required.

These parasites are common and infect a great many species of vertebrates. Cases of human infection, which are rare, have been attributed to the two genera *Eimeria* and *Isospora*. In *Eimeria* the oöcysts contain four sporocysts, each of which contains two sporozoites. In *Isospora* there are two sporocysts, each with four sporozoites.

Eimeria stiedae (Coccidium cuniculi, C. oviforme) is a very common parasite of rabbits, invading the epithelial cells of the bile passages and forming small yellowish nodules in the liver. Infection of the liver has been reported in about five human cases.



Fig. 91.— Eimeria stiedae. Očeyst containing four spores, in each of which two sporozoites are developing. (From Doflein after Metzner.)

The oöcysts (in the faeces) are about 40 by 20µ, oval, with a double-lined integument. Oöcysts of other species of fiimeria have been reported in human faeces, but according to Magath (1935) these were accidentally swallowed with infected meat and did not indicate actual infection.

Isospora hominis (I. belli).—Obcysts of this parasite have been reported in the faeces in about 200 human cases, 75% of which were found during the war in British soldiers from the castern Mediterranean region. Closely related if not identical species are common in dogs and cats in certain regions, invading the epithelial cells of the small intestine. The obcysts are ovoid structures with a smooth, colorless, doubly refractile wall, about 14 by 28 $\mu$ . When passed the contents are unsegmented, but within about 24 hours there appear two sporocysts about 13 by  $9\mu$  with doubly refractile walls within the original cyst wall, each of which contains four vermiform sporozoites about 2 by  $6\mu$  in size. In the reported cases the obcysts were found in the faeces only for a period of a few days. The parasite appears to be feebly (if at all) pathogenic for man and is of little practical importance.

### HAEMOSPORIDIA

The order Haemosporidia includes the following genera: Plasmodium, Haemoproteus (Halteridium), Leucocytozoon, Babesia, Theileria, and Haemogregarina. The malarial parasites are the only Haemosporidia which have been shown to cause disease in man.

Plasmodium (Laveran, 1880).—This genus is characterized by the invasion of red cells by the parasite, within which both schizogony and gametocyte formation take place; by amoeboid movements; by the production of pigment; and by the extrusion of flagellum-like processes (the microgametes) from the male sporont after the blood is taken from the animal and allowed to cool.

There are three important species which cause human malaria; *P. vivax*, the cause of benign tertian; *P. malariae*, of quartan; and *P. falciparum*, of malignant tertian (subtertian, aestivo-autumnal) malaria. *P. ovale*, a fourth species of minor importance, has recently been identified.

Infection with related species of *Plasmodium* has been demonstrated in other mammals, especially monkeys, and in birds. The following species have been identified in monkeys: *P. reichenowi*, resembling *P. falciparum*, in chimpanzees and gorillas; *P. kochi*, in African monkeys, *P. inui* and *P. knowlesi* in macacus monkeys in Borneo, and *P. pitheci* in the orang-outang (but not macacus) in Borneo, all resembling *P. vivax*; and *P. braziliense*, resembling *P. malariae*, in many Central and South American monkeys. Although in some cases it has been possible to convey monkey malaria to man (but not vice versa), the malarial parasites of man and monkeys are specifically distinct. There is no evidence that monkeys may serve as a reservoir of the human parasites.

The following species have been definitely established for birds: (Manwell, 1935): Plasmodium cantherium in English sparrows; P. circumflexum in the Juniper thrush; P. elongatum in canaries and English sparrows; P. nucleophilum in cat birds; P. praecox (relictum) in English and Spanish sparrows; P. rouxi in Algerian sparrows and canaries; and P. vaughani in American robins. Culex mosquitoes serve as definitive hosts in the case of the malarial parasites of birds. The first demonstration of the life cycle of the malarial parasite (Ross, 1898) was in the case of P. praecox of birds. Grassi and Bignani then proved the occurrence of a similar cycle for the organism of human malaria in Anopheles mosquitoes. Final proof was brought in 1900 when infected mosquitoes were sent from Italy to London where, by biting, they infected two persons (Sambon and Low).

Hacmoproteus (and Leucocytozoon) differs from Plasmodium chiefly in that schizogony occurs in the endothelial cells of the capillaries. The gametocytes develop in the red cells, producing pigment and exflagellating like Plasmodium, and they undergo a similar cycle of development in the insect vector. These parasites occur in birds and some cold blooded vertebrates. When full grown the gametocytes are looped about the nucleus of the red cell like a halter. The first observation of fertilization of the macrogamete by the "flagellum" (MacCallum, 1897) was in the case of a Haemoproteus infection of crows.

Life History. Cycle in Man. 1. Asexual Cycle (Schizogony).—The minute falciform parasite (sporozoite) is introduced into the subcutaneous tissue or blood stream by the bite of an infected *Anopheles* mosquito through a minute channel in the hypopharynx.

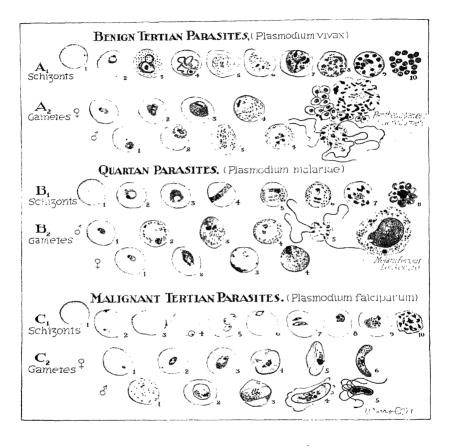
It is not necessary that the parasite be injected directly into the blood stream; successful experimental inoculation has been accomplished by allowing a mosquito to bite through the skin over a blister. It has been generally assumed (largely on the basis of an unconfirmed observation of Schaudinn) that after entering the blood vessel, the sporozoite attaches itself to a red cell into which it quickly penetrates. Some doubt as to this has been raised by the observations of Boyd and Stratman-Thomas (1034), who found that after heavy experimental infection by bites of 15 infected mosquitoes, the blood did not become infectious for a second individual (by transfusion) until the ninth day (parasites being found in films on the eleventh day). This suggests that the sporozoites may undergo a preliminary period of development in the tissues before invading the blood, or at least before beginning active multiplication. (Rowley-Lawson believes that the parasite remains extracellular, adhering to the surface by loop-like pseudopodia and migrating from one cell to another, but this view is not generally accepted.)

The parasite (trophozoite) after it has entered a red cell assumes a somewhat round or irregular shape and progressively enlarges, forming from the disintegrated haemoglobin many fine granules of pigment (haemozoin, formerly regarded as melanin). As it approaches maturity (after 48 to 72 hours, according to the species) it fills the greater part of the cell. The pigment which has been scattered becomes clustered in the center of the parasite. The nuclear chromatin, which has been relatively compact, divides into from 8 to 32 minute particles which become scattered through the cytoplasm. About these as centers the cytoplasm of the parasite (schizont, merocyte) divides into small spore-like bodies (merozoites). The merocyte then ruptures, liberating the merozoites into the plasma. These seek out and penetrate fresh red cells, and the cycle is repeated.

Multiplication in geometric progression goes on until after about two weeks (incubation period) a sufficient number of parasites has been produced to cause clinical symptoms. It is estimated that there must be at least 200 per cmm. or about a billion in the entire body. As a rule there is a tendency for the parasites to develop at nearly the same rate and to fall gradually into (one or) two groups, all members of which show about the same stage of development. Sporulation of all the parasites in the group thus occurs at about the same time. The onset of the malarial paroxysm corresponds to the time of sporulation and is attributed to the sudden liberation into the blood of toxic substances from the disintegrated red cells.

2. Sexual Cycle.—Sexual forms (gametocytes) appear in the blood at varying intervals after fever has started, perhaps because conditions become less favorable for continued multiplication. They may be present from the first in benign tertian, after a week in aestivo-autumnal, and may appear only after several months in quartan. They probably develop from pre-existing asexual parasites. The period of growth is about double that of the schizonts, but they probably do not become mature and infective until after a week or ten days. The life of a gametocyte has been estimated at from 30 to 60 days. The female macrogametocyte tends to be larger, stains more deeply blue, and contains more pigment but less chromatin than the male microgametocyte.

Sexual Cycle in the Mosquito (Sporogony).—When blood containing gametocytes is taken into the stomach of a suitable Anopheles mosquito, the microgametocyte undergoes a process of "exflagellation." This can be observed in fresh moist blood films at room temperature. The pigment shows very active movement. Long, slender, bulbous-tipped flagellum-like processes are gradually extruded (the microgametes). These show active lashing movement, and finally break away and swim about until they find a macrogametocyte. The latter, after undergoing a nuclear reduction with the formation of polar bodies, becomes a macrogamete. It is then penetrated by a single microgamete which fuses with the nucleus, forming the zygote. The zygote (called at this stage a vermiculus or oökinete) becomes capable of worm-like movement which enables it to bore through the wall of the mosquito's stomach. It stops just under the delicate outer layer where after 3 to 4 days it becomes encapsulated to form an oöcyst. The latter grows into a rounded wart-like protuberance 50µ in diameter on the outer



### DESCRIPTION OF PLATE OF MALARIAL PARASITES

(Schematic)

### Benign Tertian Parasites

- A1. Trophozoites and Schizonts. 1. Normal red cell. 2. Young ring form. 3. Amoeboid or figure-of-eight form showing Schüffner's dots. 4. Amoeboid form showing increased chromatin (24 to 30 hours). 5. Segmentation of nucleus. 6. Nuclear halves further apart, red cells enlarged and pale. 7. Further division of nucleus. 8. Unusual division form. 9. Typical merocyte. 10. Rupture of merocyte liberating merozoites.
- A2. Female gameles. 1. Young form showing solid instead of ring-form staining. 2. Half-grown form. 3. Rapidly growing form with compact nucleus and clear vacuolated zone. 4. Full grown macrogametocyte showing eccentrically placed chromatin and much pigment in deep blue stained protoplasm. Male gameles. 1. Young form similar to female one. 2. Half-grown form showing central chromatin. 3. Full grown microgametocyte showing large amount of centrally placed chromatin with light blue protoplasm surrounding. 4. Division of chromatin occurring in microgametocyte and developing in wet preparation. Note.—Chromatin division in gametocytes does not take place until blood is withdrawn. 5. Spermatozoon-like microgametes developing from the microgametocyte. This only occurs in wet preparations or in the stomach of the mosquito. "Parthenogenetic macrogamete": this object, supposed by Schaudinn to be a parthenogenetic female, has since been interpreted as two parasites (schizont and macrogamete) in a single red cell.

### Quartan Parasites

- B1. Trophozoites and Schizonts. 1. Normal red cell. 2. Young ring form. 3. Older ring form. 4. Narrow equatorial band. 5. Typical band form. 6. Oval form showing division of chromatin. 7. Early stage merocyte. 8. Daisy form merocyte.
- B2. Male gametes. 1. Young solid form. 2, 3, 4. Developmental stages microgametocytes. 5. Flagellated body in wet preparation showing microgametes developing from microgametocyte. Female gametes. 1. Young oval form. 2. Somewhat older stage. 3 and 4. Mature macrogametocytes (same as benign tertian).

### Malignant Tertian Parasites

- C1. Trophozoites and Schizonts. 1. Normal red cell. 2, 3, 4, 5, 6. Young ring forms. These are hair-like rings and are the only forms besides crescents to be found in the peripheral blood. In very heavy infections or in smears from spleen the following forms are found. 7. Beginning division of chromatin. 8 and 9. Further division. 10. Merocyte.
- C2. Female gametes. 1. and 2. Young macrogametocytes. 3. Older stage. 4. Development in red cell. 5. and 6. Fully developed female crescents showing clumping of pigment and rich blue color. Male gametes. 1. and 2. Developing forms. 3 and 4. Fully developed microgametocytes. 5. Flagellated body developed in wet preparation.

surface of the stomach wall. By repeated subdivision of the nuclear chromatin and subsequently of the cytoplasm there are formed great numbers of minute falciform sporozoites. The number may vary from several hundred to 10,000 in a single cyst, and there may be 500 cysts in the stomach of a single mosquito. When development is complete the cyst ruptures and frees the sporozoites in the body cavity of the mosquito. Many of these migrate into the salivary glands, and thence by way of the venenosalivary duct in the hypopharynx they are introduced into the circulation of the person bitten by the mosquito and start an asexual cycle. The mosquito is thus the definitive host, and man is the intermediate host. The mosquito suffers no evident injury from the infection; its life is not shortened, nor is its fertility lowered.

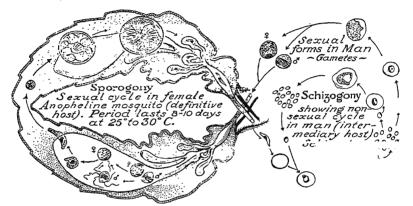


FIG. 92.—Sexual (sporogony in mosquito) and nonsexual (schizogony in man) cycles of the malarial parasite. The sporogony diagram at the left shows in lower portion the fertilization of the female gamete by the microgamete. The vermiculus stage of the zygote is shown boring into the walls of the mosquito's stomach later to become the more mature zygote packed with sporozoites as shown in the upper diagram of the developmental processes in the mosquito's stomach.

The duration of the life cycle in the mosquito varies with the temperature and the species of parasite (see table). Below 18°C. little or no development occurs, but the parasites in the mosquito may survive even ice box temperatures. Once sporozoites have developed, a mosquito may remain infectious for three months and may infect many individuals, since only a part of the parasites are discharged with each bite.

Effective Mosquito Hosts.—Of the various species of Anopheles (over a hundred), only a minority have been found capable of transmitting malaria. The possibility of transmission has been proved in about 38 species. Of these only a relatively few (12 to 15) are of much practical importance. The various factors which determine this are discussed in Chap. XXIII, and the most important species are described. There is no evidence of hereditary transmission of the parasites in the mosquito.

Species of Malarial Parasites.—Plasmodium vivax, the cause of benign tertian malaria, has a cycle of development of 48 hours. In fresh unstained preparations taken at the time of the paroxysm or shortly afterward, the young trophozoite is seen as a round or oval body, slightly more refractile than the substance of the red cell, whose outline can not be differentiated distinctly from the red cell. They are about one fifth the diameter of the red cell and are best recognized by noting their amoeboid movements. In about eighteen hours fine brownish pigment granules appear and make the parasite more distinct. After twenty-four hours the lively motion of the pigment, the projection of pseudopod-like processes and the pallor and swelling of the infected red cell make recognition easy. When about thirty-six hours old, the trophozoite having matured and the nucleus having divided to form a schizont, the amoeboid movement ceases. The pigment tends to clump into one or two compact masses, and one can recognize small, oval, highly refractile bodies within the sporulating parasite.

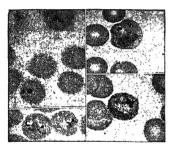


Fig. 93.—Tertian malarial parasites, one red cell showing malarial stippling. (Todd.)

Fig. 94.—Aestivo-autumnal malarial parasites; small ring forms and crescents. (*Todd*.)

The gametocytes do not show amoeboid movements but possess abundant pigment which is actively motile in the male and non-motile in the female. The male gametocyte is more refractile, is rarely larger than a red cell, and shows yellow-brown, short, rod-like particles of pigment. About fifteen minutes after making a fresh preparation, exflagellation occurs, as already described. The female gametocyte is larger than a red cell, is rather granular, and has more abundant dark brown pigment than the male.

In stained preparations (Wright, Giemsa) the young trophozoites appear as small, oval, bluish-staining rings about one fifth the diameter of the red cell. One side of the ring is distinctly broader than the other which contains a fine red dot of chromatin—giving the parasite the appearance of a signet ring. These rings are seen in the blood at about the beginning of the sweating stage of the paroxysm. It is rare to find two chromatin dots or more than one parasite in the same cell. When about 24 hours old the parasite contains much pigment, has an amoeboid or double figure-of-eight contour and is about three-fourths the diameter of a red cell. The infected red cells are about one and a half times as large as normal, show a pale, washed-out appearance, and (in heavily stained films) show many fine, reddish yellow dots (Schiffner's granules). These, practically, are peculiar to benign tertian infection. A few hours before completion of the forty-eight hour cycle the sporulating parasite shows about sixteen small

round bluish bodies containing a chromatin dot, irregularly distributed over the area of the merocyte.

The young gametocytes show a thicker blue ring, and have the chromatin dot in the center of the ring. The half-grown gametocytes show more abundant pigment than do trophozoites of equal size, and they are round or oval in shape and not amoeboid. The full grown gametocytes have the pigment distributed and the chromatin in a single aggregation—just the opposite of the full grown non-sexual parasites. The male gametocyte stains a light greyish blue and has a very large amount of chromatin, usually centrally placed. The female gametocyte stains a deeper pure blue and has only one tenth as much chromatin as plasma, with the chromatin often clumped at one side.

P. vivax is the most wide spread of the malarial parasites, occurring throughout the tropics and subtropical regions and in extensive areas in the temperate zone. It has occurred as far north as southern Sweden and England and the Great Lakes of North America.

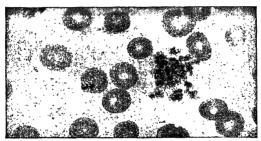


Fig. 95.—A cluster of blood-platelets and two platelets lying upon a red cell and simulating malarial parasites (× rooo). (Todd.)

P. malariae, the parasite of quartan malaria, has a cycle of 72 hours. It differs from P. vivax in the following points. In fresh preparations the young trophozoites are more clear cut and waxy in appearance. Amoeboid movements are only slight. In older forms the parasite tends to assume an oval or band shape. The pigment is abundant, coarser and blacker, and often peripherally arranged. The red cell is but little altered. The 6 to 12 merozoites in the sporulating schizont form an even circle around a dense mass of black pigment, like the petals of a daisy. The gametocytes are somewhat smaller.

This parasite is relatively rare, being found chiefly in scattered areas in the Mediterranean region and in southern India, Ceylon, and the Malay peninsula. It also occurs in tropical Africa and in America. The disease clinically is the mildest of the three types, but it is the most resistant to treatment and most prone to late relapses. It is the most frequent cause of malarial nephritis.

P. falciparum, the cause of malignant tertian malaria, has a cycle of development of about 48 hours. However, this is variable, and there is not as much tendency to simultaneous development of the parasites as is shown by the other two species. As a result sporulation is often protracted or continuous, and the fever is irregularly remittent or continuous or the paroxysms, if they occur, are protracted.

The young trophozoites are difficult to detect in fresh preparations, appearing early in the "hot" stage of the attack as minute crater-like dots about one sixth the diameter of the red cell. However, they show active amoeboid movement. They are usually in the periphery of the red cell. It is common to find several parasites in the same cell. They gradually enlarge to become about a third the diameter of the cell, and occasionally a few fine brownish pigment granules may appear. At this stage the infected red cells disappear from the peripheral blood except in rare instances in very severe infections.

In severe infections there may be extraordinary numbers of parasites present, many in every field and up to eight in a single cell. It has been estimated that there may be three trillion in the entire body.

In stained films the young parasites appear as hair-like rings, often with two chromatin dots on one side of the ring. They often appear as if plastered on the periphery of the cell, or as if they had destroyed a rounded section of the rim of the cell. Rarely they may be bacillary in shape and show no vacuole, being recognizable by the red chromatin dot. The infected cells may show diffuse basophilic staining and distortion. Schüffner's dots do not occur, but in heavily stained films Maurer's dots may be seen as coarse, scattered, deep brick-red dots or clefts. It is not possible to identify the species of parasite if the early ring forms alone are present. However, if fine rings are present on one examination, and if twelve hours later they show definite thickening, P. falci parum can be excluded, as this species nearly always disappears from the circulation by the time this stage is reached.

The infected red cells tend to agglutinate and to adhere to the walls of the capillaries of the internal organs in which they may form plugs and cause grave symptoms attributed in part, at least, to infarction of these organs. In sections of tissue from the brain, liver, spleen and bone marrow from such cases (prepared in the usual way by formalin fixation and haematoxylin and eosin staining) the parasites are usually revealed by clusters of fine brownish-black pigment grains within the red cells in the capillaries and smaller blood vessels. Such an appearance is diagnostic of malarial infection with P. falci parum. Unless special methods of staining and fixation are employed, the parasites themselves can not be seen in such tissues.

In blood obtained by puncture of the spleen (caution!) or bone marrow, the older trophozoites and schizonts may be found. The infected red cells become shrunken and brassy in color. The schizonts occupy one half to two thirds of the red cell and show usually about sixteen irregularly scattered merozoites with one or two compact masses of dark brown pigment.

The crescent-shaped gametocytes are characteristic of this species and can often be found in the blood after a week of fever. For sex differences see table. In some cases young gametocytes may be ovoid rather than crescentic, and in fresh preparations they tend to become swollen and ovoid as flagellation occurs.

P. falciparum is widespread throughout tropical and subtropical regions, but is relatively rare in the temperate zone. It is the most dangerous type of malarial parasite, sometimes giving rise to "pernicious" attacks which may be quickly fatal. However, (if reinfection is avoided) it dies out more quickly and is more susceptible to treatment than the other species.

P. ovale has a cycle of development of 48 hours. The trophozoites resemble those of *P. malariae* in showing but little amoeboid movement, and tend to assume a bandshape. The pigment is granular and blackish brown. The schizonts are smaller than a red cell and contain from 8 to 12 merozoites. The gametocytes are oval and smaller

than a red cell. The infected red cells are slightly swollen, are oval in shape (hence the specific name), have ragged fimbriated margins, and even in the early stages of development of the parasite, contain numerous Schülfner's dots. Unlike the other species, the febrile paroxysms tend to occur between noon and midnight. Thus far this species has been reported only in cases from west Africa (Congo, Nigeria). The infection is mild and short lived. It has been used for therapeutic inoculation in cases of general paresis in which the more severe reactions caused by the other species of parasites were contraindicated because of the age or physical condition of the patient.

Laboratory diagnosis of malaria depends upon demonstrating the parasites in blood films. If they are sparse, thick films should be stained with Giemsa's stain (see p. 308). As the parasites are somewhat distorted in such preparations, to identify the species thin films should also be stained.

Concentration method of Bass and Johns.—This procedure is more tedious than the preparation of thick films, but the parasitized red cells are effectively concentrated and the red cells and parasites are perfectly preserved and stain as well as in ordinary thin films. Blood (5 or 10 cc.) is mixed with a minimal quantity of oxalate or sodium citrate solution and centrifugalized at 2500 R.P.M. The upper layers of red cells,

## UNSTAINED SPECIMEN (FRESH BLOOD)

ONSTAINED OF ECIMEN (TRESH BLOOD)			
	P. vivax (benign tertian)	P. malariae (quartan)	P. falciparum (malignant tertian) (aestivo-autumnal)
Character of young trophozoite.	Indistinct amoeboid outline. Hyaline. Occupies 1/4 to 1/3 of a r.b.c.	More dense and less amoeboid than P. vivax. Frosted glass appearance of disclike parasite. Size same as P. vivax.	Small crater-like dots ½6 the diam. of r.b.c. Common to find 2 or more parasites in 1 cell.
Character of growing tropho- zoite.	Highly amoeboid, ("vivacious"). Fine yellowish brown pigment granules.	Very sluggish movement. Forms more compact in outline. Pigment in coarse black grains or chunks which may be in sluggish motion.	Older trophozoites rarely seen in peripheral blood. Fine black pigment scattered evenly in cytoplasm.
Character of mature schizont.	Amoeboid outline but no movement. Yel- low-brown pigment which is motile early in schizogony.	Oval in shape. Coarse black pigment masses moving slug- gishly in early schiz- ogony.	Not seen in peripheral blood except in mori- bund cases. Scanty chunks of fine black pigment.

## STAINED SPECIMEN

P. vivax

P. malariae

P. falciparum.

fected cell.

normal red cell.

Character of in- Larger and paler than About normal size and Usually normal. Late in color. (Cell may be contracted.)

disease some distortion. Cells with old parasites shrunken and brassy.

Stippling of infected red cells. 'Schüffner's dots' characteristic when found. First appear when trophozoite has almost matured. Best seen in schizont stage.

None. (Ziemann's stippling brought out only by special staining methods.)

Basophilic stippling may occur late in the disease Stephens and Christopher's or Maurer's dots seen occasionally. (See text). Same cell may also show basophilic stippling.

tropho- Signet ring forms up to 1/3 Young zoite (early rings).

See footnote.

Caution:

diameter of red cell. Single vacuole. No pigment. Accole' forms rare. Schuffner's dots usually absent. Occasional double infection of red cell.

Signet rings slightly smaller and denser than P. vivax. Large deep red chromatin dot. Cytoplasm deep blue. Fine black pigment grains occasionally seen. Double infection of red cell rare.

Smallest of the 3 species. 15 to 16 diam. of r.b.c. Cytoplasm thin and hair like, light blue, much chromatin. No pigment. Bacillary forms occur. Accole' or marginal forms common. May find up to 8 parasites in the same red cell.

zoite (older rings).

Growing tropho- Irregular amoeboid outline with fine strands of cytoplasm. Often 2 or more vacuoles. Fine vellow-brown pigment grains. Schuffner's dots occasionally.

Oval, band, ribbon and comet forms. Compact, the single vacuole soon disappears. Black coarse pigment in masses and coarse grains, more abundant about periphery. The most pigmented species.

Ring 1/4 diam. of red cell. Often 2 chromatin dots in a single ring. Dustfine black pigment scanty, evenly distributed in thin ring.

Schizont.

Invaded red cell pale, enlarged. Shows Schüffner's granules. Contour very irregular. Fine yellowbrown pigment grains. Two to rounded chromatin masses.

Nearly fills the unaltered cell. Rounded, deeply pigmented. definitely outlined, compact parasite, with deep blue cytoplasm and 6 to 8 chromatin masses.

Only seen in peripheral blood in moribund cases. Occupies 1/2 to 3/3 of red cell. 8 to 12 dots of chromatin.

Merocyte (sporulating schizont) "Rosette."

Almost fills distended red cell. Irregular grapelike cluster. Yellowish brown pigment massed eccentrically. Schüffner's dots may be present.

Nearly fills a normal-sized Not seen in peripheral red cell. Typical "daisy" form, with merozoites arranged more symmetrically around a central darker pigment mass.

blood except in moribund cases. Fills 1/2 to 26 of red cell. Pigment mass eccentric, dark brown.

## STAINED SPECIMEN (Continued)

	P. vivax	P. malariae	P. falci parum.
Number of merozoites.	12 to 24. Usually 14 to 16.	6 to 12. Usually 9	8 to 34. Usually 14 to 16. (Up to 32 in culture.)
Microgametocyte (male).	Spherical, 10 to 12µ. Cytoplasm pale greyish or greenish blue. Chromatin abundant, diffuse, pale pink, central or in a band. Scattered yellowish brown pigment.	Similar to P. vivax but smaller. Central chunks of black pigment. The most pigmented of all malarial parasites.	Short, stout, sausage- shaped. Pale blue cyto- plasm. Chromatin pale pink, diffuse, abundant, central. Pigment scat- tered, overlies the nu- cleus. A little red cell cytoplasm often seen in concavity of crescent.
Macrogametocyte (female).	Spherical, fills distended red cell. Cytoplasm deep blue. Chromatin compact, deep red, ec- centric, with halo around it. Pigment abundant, rather coarse.	Similar to P. vivax but smaller. Abundant brownish black pigment.	More pointed and longer than male. Nucleus more compact and stains more intensely red. Pig- ment clumped in coarser grains in center.
General character of blood films.	All phases of schizogony present with wide variety of amoeboid forms. Multiple infection of red cells not rare. Gametocytes early. Schuffner's dots.	All phases of schizogony. Gametocytes appear late. Sporulating forms occasionally seen at any period of cycle. Multiple infection of red cells rare. No granulation of red cell cytoplasm.	Rings and crescents only forms seen in peripheral blood. Multiple infection of red cells common. Parasites usually more abundant than in the other two infections.

Caution.—While the differential points tabulated are dependable as a rule, a diagnosis should never be made without studying a number of individual parasites. It is often necessary to observe the parasites through an entire cycle. The species can not be differentiated by means of the early ring forms alone (see text). The most serious mistake is to overlook *P. falciparum*, either in single or mixed infections. The ring forms are often detectable in the peripheral blood only during a small portion of the cycle.

which include practically all those containing parasites, are pipetted off into a smaller tube and again centrifugalized. This is repeated once or twice more if necessary, and thin films from the upper layers of the final sediment are made and stained in the usual way (Wright or Giemsa).

Blood should be examined several times a day throughout a cycle, as the trophozoites of  $P.\ falciparum$  may otherwise be missed.

Additional differential points (motility, refractility) may be made out in fresh preparations, especially in the early stages of the cycle.

Blood obtained by puncture of the spleen (a somewhat dangerous procedure) or sternal bone marrow will frequently show parasites in

## BIOLOGICAL DIFFERENCES

	$P.\ vivax$	P. malariae	P. falciparum
Extrinsic incubation (in mosquito)	Optimum 17 days. (May be retarded by adverse weather conditions.)	Optimum 35 days.	Optimum 23 days.
Optimum tem- perature for ex- ogenous cycle.	25 degrees C.	22 degrees C.	30 degrees C.
Intrinsic incuba- tion (in man)	14 to 16 days.	27 to 42 days.	10 to 12 days.
Incubation after experimental inoculation	4 to 22 days. (Depends upon the number of infected red cells injected.)	11 to 50 days.	2 to 14 days.
Gametes appear in peripheral blood.	From first day of fever onward.	Several months after first attack of fever.	122001 2 110011 01
Length of asexual cycle.	48 hours.	72 hours.	24 to 48 hours (or irregularly continuous.)

latent cases in which they can not be found in the peripheral blood. See also Provocative procedures, page 454.

Cultivation by Bass's method (see p. 856) has not proved to be a practical diagnostic procedure.

The blood shows a haemolytic type of anaemia which may be extreme. The red cell count may be reduced by a half million or even a million during a single paroxysm. During the paroxysm there is usually a leukocytosis. In the intervals there is a leukopenia and an increase in monocytes, often to 15% or 20%. This may be absent in native races long exposed to endemic malaria. The presence of pigmented phagocytes in blood films is diagnostic of malaria. Such "melaniferous leukocytes" are of rare occurrence, and in blood which contains them parasites can usually be found in thick films.

Reticulocyte crisis.—If quinine is administered to a patient with malarial anaemia, there is usually a rise in the percentage of reticulocytes which reaches a maximum within 4 to 7 days. This is said to be specific for malaria and not to occur in other types of anaemia.

Henry's flocculation test has not proved to be of much practical value. The reaction was first thought to be due to a specific precipitation between antibodies in the serum and melanin (antigen), but it is merely a non-specific precipitation of the serum globulin

## CLINICAL DIFFERENCES

	P. vivax (benign tertian)	P. malariae (quartan)	P. falciparum (malignant tertian)
Onset of fever.	Usually sudden.	Usually sudden.	Insidious or sudden. Chills may be absent. Occasional severe case with normal or sub- normal temperature.
Duration of fever paroxysm.	6 to 8 hours.	4 to 6 hours.	12 to 36 hours or more.
Duration of first untreated at- tack.	2 to 4 weeks.	I to 2 months.	10 days to 2 weeks.
Type of temperature curve. First attack.	Intermittent or remittent for first 3 days, then quotidian for ten days, then tertian. Relapses usually tertian from onset.		No characteristic temper- ature curve. Quotidian periodicity more com- mon than tertian.
Enlargement of spleen.	Early, first few days.	Early, first few days.	Often much later than in the other types.
Clinical types.	Attacks of "chilis and fever" followed by sweating.	Attacks of "chills and fever" followed by sweating. Quartan perio- dicity.	Usual type: attacks of quotidian, tertian or continuous fever, with or without sweats. This may change suddenly into a pernicious type (see text).
Relapses.	May occur in 2 to 10 weeks and again in 6 to 9 months. Rarely up to 3½ years after the original infection.  More resistant to quinine than P. falciparum.	The most persistent of all. Relapses have occurred 6 years after first infec- tion. The least suscep- tible to quinine.	Relapse in untreated cases in about 2 weeks. Relapse rare after 0 months. Longest period recorded 1½ years. Most susceptible to quinine. Atebrin more effective than quinine.
Sequelae.	Апаетіа.	Anaemia. Nephritis in certain endemic areas.	Anaemia. Various neu- rological disturbances in pernicious cases. Black- water Fever may come on after a year's resi- dence in an endemic area.

which is much increased in quantity during the acute stage of the disease. (For discussion see Greig et al., 1934.)

# Miscellaneous Questions Concerning the Malarial Parasite in Man

- 1. Multiple Infections.—Quotidian paroxysms may perhaps be produced at times by infected mosquitoes biting on successive nights so that one crop will mature and sporulate twenty-four hours before the second. However, in the first attack of (tertian) malaria, whether spontaneously acquired or experimentally produced by one bite of a single infected mosquito, the temperature during the first few days of fever is continued or irregularly remittent. Then, as the parasites become assembled into groups, quotidian paroxysms of intermittent fever occur, "double tertian" infection. Later one group of parasites often dies out, and the fever becomes "single tertian." In relapses the fever is intermittent and tertian from the onset. In quartan malaria the infection is usually single, but "double" and "triple" infections may occur. In aestivoautumnal infection the paroxysms, if present, may be quotidian or tertian.
- 2. Mixed infection is a term applied to the simultaneous presence of two or more species of parasites in the same individual. Mixed infections with malignant tertian and benign tertian are the most common, next malignant tertian and quartan, while cases with benign tertian and quartan are quite rare. All three species have been found in the same individual. In double infections of malignant tertian with one of the other species, the former parasites are often absent in ordinary blood films and yet may be transmitted by inoculation. This is of great practical importance in therapeutic inoculations with malaria, since such double infections have a grave prognosis (mortality 50% and higher).
- 3. The occurrence of cases of *congenital malaria* has been demonstrated, but they are extremely rare.
- 4. Immunity to malaria has been studied experimentally in birds and monkeys. After a varying period of active infection during which parasites are numerous in the blood, resistance develops, symptoms subside, and parasites can no longer be found in blood films. Such animals for some time can not be reinfected with the same strain of parasite. However, it can be shown that parasites persist in the blood in small numbers (latent malaria), because inoculation of such blood into normal animals produces the disease. As soon as complete cure is effected (after from several months to a year or two) and the blood is no longer infectious, immunity disappears and the animal can then be reinfected. Such immunity which depends upon a persistent latent infection is termed premunition.

It has been shown that the same conditions exist in the case of human malaria. There is relatively little natural immunity to malaria (at least to malignant tertian), although occasional individuals appear to be resistant. In a heavily infested region practically all the native children are infected shortly after birth and are continuously exposed to reinfection. The mortality among the young children is very high, but the survivors gradually develop a resistance to the parasite so that they become free from fever and clinical symptoms, although the spleen is much enlarged and all show many parasites in the blood. As they grow older the number of parasites diminishes, and in adults in about 50% of the cases it is no longer possible to find parasites in blood films. The same course is seen in susceptible adults who move into an infested district.

This immunity is effective only toward the species with which the individual is infected, and to a large extent only toward the infecting strain of that species. This

perhaps explains why maximum protection is obtained only after about fifteen years' residence in a malarial district. The immunity is maintained only by continued reinfection, for if a native leaves an infected district he soon loses his immunity.

The immunity appears to be due to a proliferation and increased activity of the reticulo-endothelial cells, especially of the spleen, which engulf and destroy the infected red cells as well as free parasites. This stimulation is possibly a manifestation of a true hypersensitive reaction to the foreign protein of the parasite, as it disappears when the parasites are completely eliminated. Splenectomy greatly increases the susceptibility of monkeys to malaria, as does blockade of the reticulo-endothelial system in birds. Serum antibodies possibly exist, but no conclusive proof of this has been obtained, and the blood serum has no protective power.

- 5. Latent malaria is a term applied to persisting infections which give rise to no clinical symptoms and in which parasites usually can not be demonstrated except by inoculation of large amounts of blood.
- 6. Relapses are recurrent attacks of clinical malaria in individuals who have had latent malaria, with the reappearance of parasites in the blood, usually in large numbers. The term "recrudescence" is sometimes used for attacks occurring within two months of the original infection and "recurrences" for attacks occurring after an interval of seven or eight months. The late recurrences are a characteristic feature of benign tertian malaria, but do not occur in malignant tertain. Gametocytes are much more numerous during a recurrence than in the primary attack (James, 1936). Relapses are highly characteristic of all types of malarial infection, being most marked in quartan and least in malignant tertian (see table p. 452).

Relapses may occur without obvious cause but are apt to follow any condition which lowers the general resistance of the body. Among these may be mentioned exposure to cold and wet, to intense sun light, excessive fatigue, alcoholic, dietetic or venereal excesses, an intercurrent illness, a serious accident (fracture), a surgical operation, and child birth. Persons returning home from the tropics often experience relapses as they approach the cooler climate of the temperate zone. It has been well stated that the old resident of the tropics owes his condition of health rather to education than to acclimatization—experience has taught him discretion.

It is now believed that relapses are due to failure of the defensive forces of the body to restrict the multiplication of the parasites to negligible proportions, as they do during the latent stages of the infection (Ross, Bignami). There is no proof of the existence of special resistant asexual forms. Schaudinn's theory that female gametocytes may undergo parthenogenetic multiplication has been disproved completely. Attempts to transmit malaria by inoculation of blood containing only gametocytes have been uniformly unsuccessful.

- 7. Provocative measures to induce a relapse with a reinvasion of the blood by malarial parasites are occasionally employed for diagnostic purposes. Among these may be mentioned fatigue, refrigeration, exposure to sun light, administration of small doses of quinine for ten to fourteen days, or of berberine sulphate, intravenous injections of typhoid vaccine, and subcutaneous injections of epinephrin (usually regarded as the most effective procedure). Videla (1934), however, has reported successful results in four of six cases in which usual methods had failed, by daily intravenous injections of 10 cc. of 10% calcium chloride solution for two or three days.
- 8. Malarial Toxin.—The nature of the toxic material liberated at the time of the paroxysm is not known. Brown suggested that the pigment produced by the parasite

acts as an haemolysin. The number of red cells destroyed in a paroxysm is far greater than the number actually invaded by the parasites. The pigment might also injure the phagocytes which engulf it. Abrami regarded the paroxysms as an anaphylactic reaction precipitated by the liberation of the malarial (foreign) protein at sporulation. This occurs some hours before the cold stage, which he regards as a manifestation of anaphylactic shock. A leukopenia and a lowering of the blood pressure preceding the paroxysm are considered evidences of a haemoclastic crisis.

9. Types of Malignant Tertian Malaria.—The usual types are: (a) the intermittent type with tertian or quotidian paroxysms, differing from benign tertian chiefly in the more protracted febrile periods and in usually greater prostration and malaise in proportion to the degree of fever; (b) the more frequent remittent type, in which the paroxysms merge; (c) the "bilious remittent" type, of greater severity, characterized by vomiting of bile stained material, by jaundice, and the development of severe anaemia. Cases showing marked prostration, delirium and subsultus have been termed "typhoid remittent." Although patients with malaria of these clinical types commonly recover, at any time, even in apparently mild attacks, there may be an abrupt and extreme increase in the severity of the symptoms (accès pernicicux) which, if not effectively treated, may be fatal within one to three days. In some cases the course is pernicious from the start. Most "pernicious" cases of malignant tertian malaria may be grouped as cerebral forms or algid forms.

In the cerebral forms the parasites are found in enormous numbers in the brain capillaries. Clinically the predominant features may be hyperpyrexia, delirium, coma, psychoses of various types which may simulate acute alcoholism, convulsions (in children), signs of meningeal irritation, amblyopia, or apoplectic phenomena (hemiplegia, aphasia, etc.). In the algid forms the parasites tend to accumulate in the capillaries of the gastrointestinal tract and abdominal organs. There is usually profound prostration with a tendency to fatal syncope, and extreme coldness of the skin with a high internal temperature. Several clinical types may be distinguished: gastric, with incessant vomiting; choleraic, dysenteric, with blood in the watery stools; haemorrhagic or purpuric. A grave haemolytic anaemia may develop with great rapidity. "Pulmonary" and "cardiac" types have also been described.

It is not known why in one case sporulating parasites should plug the capillaries of the central nervous system, while in another case the damage is done to the intestinal mucosa, pancreas, or lungs. These pernicious manifestations of malaria should always be kept in mind when a case of sudden cerebral involvement or acute abdominal disease is found in a patient in a malarious country, and a blood examination should be made promptly.

It has been suggested that the pernicious attacks may be due to: (1) The very large number of red cells infected and destroyed by the malarial parasites; (2) the large amount of toxic material liberated at sporulation by such a large number of disintegrating merocytes; and (3) the plugging of the capillaries of internal organs by the sporulating parasites. This may arise (a) because the sporulating parasites are too large to pass through the capillaries, or (b) their ovoid shape prevents their passing through the capillaries, whereas the flattened benign parasites can do so by infolding (Bass); or (c) from the tendency of malignant tertian parasites to agglutinate; or (d) from degenerative changes or distension with pigment of the endothelial cells lining the capillaries.

ro. Action of Drugs on Malarial Parasites.—Quinine acts specifically on the trophozoites of all species of malaria (much more actively on malignant tertian than on benign tertian), and on the gametocytes of benign tertian and quartan, but has little effect on the crescents. The crescents are also resistant to atebrin, but this is an effective substitute for quinine with the other forms, being regarded as usually less toxic and more efficient in preventing relapse. However, cases of psychosis following atebrin administration have been reported, and smaller dosage has been suggested. Plasmoquine is effective for all forms of benign tertian and quartan parasites. It has no effect on malignant tertian trophozoites, but it is the only drug which affects the crescents, preventing exflagellation and rendering the patient non-infectious to the mosquito. As toxic symptoms have been reported following treatment with either atebrin or quinine combined with plasmoquine, it appears that any routine treatment containing plasmoquine is to be condemned.

The usual prophylactic dose of quinine (5 to 10 grains per day), although effective in preventing clinical symptoms of malaria, does not prevent infection. An acute attack is likely to occur if treatment is not continued for several weeks after leaving an endemic area.

The mechanism of the action of quinine is not definitely known. It is commonly assumed that it acts directly on the parasites, since it not only causes them to disappear promptly from the blood but appears to produce degenerative changes (abnormal staining reactions) in those which can be found. However, the parasites are much more resistant to quinine solutions in vitro than would be expected if its action were directly parasiticidal, and some believe that its main action is to stimulate the activity of the phagocytes. In animal malaria quinine loses its effectiveness if the activity of the reticulo-endothelial cells is experimentally depressed or abolished.

- 11. Malarial (Endemic) Index.—Various procedures have been employed to estimate quantitatively the prevalence of malaria in a region in which it is endemic. The percentage of mosquitoes which are infected may be determined by dissection. The parasitic index (Ross) is the percentage of individuals who show parasites in the blood, and obviously underestimates the incidence of infection in varying degree. The splenic index is the percentage of individuals who show enlargement of the spleen by palpation, while the endemic index includes all who show either parasites or splenomegaly. The most dependable results are obtained from observations on children from 2 to 10 years of age. Usually there is a fairly close correlation between splenic enlargement and demonstrable infection at this age. A region in which splenic enlargement is found in less than 10% of the cases is "healthy"; enlargement in from 10% to 25% indicates moderate endemicity; from 25% to 50%, high endemicity; and from 50% to 90% or higher, hyperendemicity. In interpreting such findings one must consider many other factors which can not be covered in a brief discussion. (See Boyd, Introduction to Malariology, Harvard University Press, 1930.)
- 12. Therapeutically induced malaria (or some other form of artificial fever) is now generally recognized as the most effective if not the only effective method of treating general paresis, and it is often used in other types of neurosyphilis. Benign tertian malaria is used in most cases. Quartan malaria may be given to patients who are resistant to benign tertian, particularly to negroes, or who spontaneously recover prematurely

from an attack of the latter. Malignant tertian should never be used, at least in Caucasians, as the mortality has been very high in patients inoculated with it, accidentally or otherwise. It has been used successfully in negroes. Subjects for inoculation should be selected with care. Malaria should not be given to patients who are debilitated or cachectic, or who have myocardial insufficiency, advanced nephritis, cirrhosis, or other serious complicating disease.

Inoculation may be accomplished by the bites of infected mosquitoes or by the injection of infected human blood. The former procedure, widely used in Great Britain, offers the advantage that no treponemata can be inoculated along with the malaria, and that the accidental inoculation of malignant tertian can be more readily excluded. The disadvantages are that infection induced by a mosquito bite is less susceptible to quinine and more likely to relapse, and the procedure is much more cumbersome. In the United States direct inoculation of blood is extensively employed. Five cc. of blood, preferably obtained at the height of the paroxysm, are injected subcutaneously or (more effectively) intravenously. It need not be matched as to group. If an infection is not obtained, larger amounts of matched blood (up to 100 cc.) may be given intravenously. There is greater assurance of infection, and the incubation period is shorter. There is no real danger of superinfecting a patient who has syphilis with a different strain of treponemata by giving blood from a case of late neurosyphilis.

After an incubation period of usually three days to a week, the temperature becomes irregularly elevated for two or three days; then quotidian paroxysms of fever begin, and as a rule these soon become tertian if the infection is not quickly interrupted. If the patient's condition permits, the infection is allowed to continue until from eight to twelve paroxysms have occurred. Quinine is then given; 5 grains three times a day will usually stop the fever after two days, but the drug should be continued for two weeks as a precaution against relapse. Patients must be watched with great care, and quinine must be administered at once if their condition becomes serious; particularly if the blood pressure falls below 70 mm., if tachycardia becomes marked, if the haemoglobin falls materially below 50%, if the parasites become excessively numerous, or if renal insufficiency or marked jaundice develops. The urine must be watched with special care if quartan malaria is given. In severe infections it is often possible, by giving one or two doses of 2.5 grains of quinine, to change the incidence of the paroxysms from quotidian to tertian or to interrupt the fever for two or three days without terminating the attack.

Re-inoculation is usually possible during the next two or three months if the attack did not terminate spontaneously, but after a longer interval the patients become resistant to re-inoculation with the same species and remain so for about five years.

The inoculated disease (man to man) differs from the natural infection in that the fever is more irregular, the paroxysms are less clear cut, and late recrudescences are never observed.

There is controversy over whether the improvement is due solely to the hyperpyrexia or whether it is due also to a stimulation of the production of phagocytes by the reticuloendothelial cells.

13. Blackwater fever is a disease characterized by a sudden onset with chill, fever, prostration, pain in the loins, haemoglobinuria, jaundice, the rapid development of a severe anaemia (down to 500,000 red cells), and (in fatal cases) anuria. The mortality ranges from 20% to over 50%. The pathogenesis is still uncertain. The disease is practically limited to individuals who have lived at least a year in areas in which malignant



Fig. 96.—
Meischer's sac from the musculature of a hog.
× 30 diameters.
(From Ostertag.)

tertian malaria is endemic and who have had repeated attacks of this infection. However, in only 50% to 70% of the cases can parasites be demonstrated in blood films during the attack. Chronic infection with P. falciparum is the essential underlying cause. In most cases some additional factor operates to precipitate an attack, such as exhaustion, chilling, alcoholic excesses, or (most frequently) the administration of quinine. In many recorded cases, however, no quinine had been administered. The blood does not give the Donath-Landsteiner reaction.

Fernán-Núnez (1936) has assembled evidence to support the theory that the attack is an acute allergic reaction to the malarial protein. He prepared an antigen by concentrating parasites by Bass and John's method and adding 0.4% formalin, and tested hypersensitiveness by intracutaneous injections of 0.2 cc. In a group of 410 individuals of white or mixed blood who had lived in an endemic area in Columbia for six months or more and who had had aestivo-autumnal malaria, he obtained 16 positive reactions (a local area of inflammation appearing

within 12 hours). After these hypersensitive cases had been sent to a non-malarial district, blackwater fever disappeared from this community, although he had observed between 50 and 100 cases during the preceding five years.

## Piroplasmidea

This suborder includes the genera *Babesia* and *Theileria*. These parasites occur in many species of mammals, but none have been demonstrated in man.

Babesia includes minute oval or pear-shaped organisms which invade and multiply within the red corpuscles and destroy them in large numbers, causing haemoglobinaemia and haemoglobinuria. They do not produce pigment. B. bigemina is the cause of Texas fever (red water) of cattle. It is transmitted by the tick Boophilus annulatus. B. canis causes malignant jaundice in dogs. B. ovis causes a similar disease of sheep ("Carceag") in the Balkans (transmitted by the tick Rhipicephalus bursa). Organisms of this kind have been thought of (but never demonstrated) in connection with Blackwater fever of man.

Theileria multiply by schizogony in the vascular endothelial cells, later invading the red blood cells in which they appear as minute bacilliform or coccoid bodies (possibly gametes). T. parva is the cause of East Coast (Rhodesian) fever of cattle in Africa, which is a very grave infection but not associated with haemoglobinuria or jaundice.

#### Sarcosporidia

These organisms (Sarcocystis) are parasitic within the striated muscle fibres of many mammals (especially pigs, mice, sheep, cattle and horses) and occasionally in birds. About six human cases have been reported. The sarcocysts (Miescher tubes) are elongated tubular bodies which distend the muscle fibres and may reach a length of 16 mm. The tubes are subdivided into numerous chambers which are filled with sickle- or oval-shaped spores (Rainey's corpuscles), 7 to  $15\mu$  long, and 3 to  $4\mu$  wide. There is an enveloping capsule for the sarcocyst which may show striations. The life history is unknown. Mice can be infected by feeding them the spores. Heavy infections may be fatal, especially to mice, but light infections are harmless. In some places more than 50% of the sheep and pigs may show infection. The parasite of sheep has been named Sarcocystis tenella; that of the ox, S. blanchardi; that of the mouse, S. muris.

## CHAPTER XIX

## FLAT WORMS

## CLASSIFICATION OF THE PLATYHELMINTHES (FLAT WORMS)

Class Trematoda			
Superfamily	Family	Genus	Species
Dana and biotomoridos	∫ Gastrodiscidae	Gastrodiscus	G. hominis
Paramphistomoidea	Paramphistomidae	Watsonius	W. watsoni
	Fasciolidae	(Fasciola	F. hepatica
		Fasciolopsis	F. buski
		(	P. ringeri
		Paragonimus	P. westermani
	Troglotrematidae	1	P. compactus
Fascioloidea.		Troglotrema	T. salmincola
	Echinostomatidae	Echinostoma	E. ilocanum
	0.1.1.1.111	(Opisthorchis	O. felineus
	Opisthorchiidae	, -	C. sinensis
	Heterophyidae	Heterophyes	H. heterophyes
		Metagonimus	M. yokogawai
	Dicrocoeliidae	Dicrocoelium	D. lanceatum
		Schistosoma	/S. haematobium
0.11			S. japonicum
Schistosomatoidea			S. mansoni
			S. intercalatum
Class Cestoda			
T) (1 : 1 1 : 1	51 1 11 1 11 11	(Diphyllobothrium	D. latum
Bothriocephaloidea	Diphyllobothriidae	Diplogonoporus	D. grandis
	Davaineidae	Davainea	D. madagascariensis
Taenioidea.	Dipylidiidae	Dipylidium	D. caninum
	Hymenolepididae	Hymenolepis	(H. diminuta
			H. nana
	Taeniidae -	(Echinococcus	E. granulosus
		Taenia	T. solium
			T. saginata

Note.—Two larval Taeniidae are found in man (Cysticercus cellulosae and Echinococcus granulosus); also two larval Diphyllobothriidae (Sparganum mansoni and Sparganum proliferum).

## TREMATODES OR FLUKES

Flukes are non-segmented flat worms, usually leaf-like in outline, rarely cylindrical. They are especially characterized by the possession of

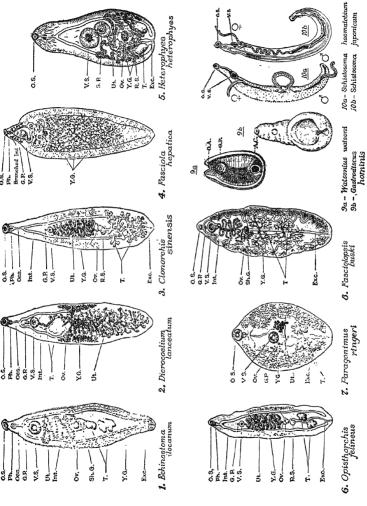


Fig. 97.—Anatomy of trematoda (flukes) of man. O.S., oesophageal sucker; Ph, pharynx; Oes, oesophagus; G.P., genital pore; V.S., ventral sucker or acetabulum; Ul., uterus; Im., intestines; Oe, ovary; Sh. G., shell gland; T. testicles; T.G., yolk glands or vitellaria; Exc., excretory pore.

suckers by means of which they attach themselves to the skin, mucosa, or other tissues of their host. With the exception of the Schistosomatoidea, all flukes are hermaphroditic, and their eggs are operculated (provided with a lid). The only other operculated eggs met with in human infections are those of the Diphyllobothriidae.

Morphology.—Flukes have two suckers which, except in the Paramphistomoidea, are quite close together: an oral sucker surrounding the mouth at the anterior extremity, and a ventral sucker or acetabulum. The intestinal tract consists of a pharynx, proceeding from the oral sucker, which bifurcates and terminates in two blind intestinal caeca. In the Schistosomatoidea the branches reunite to end in a single caecum. There is no anus.

The excretory system consists of numerous, scattered, ciliated "flame" cells from which minute canaliculi arise. These unite to form two collecting ducts which join posteriorly in the excretory vesicle which discharges through the excretory pore.

There are usually two testes, which vary greatly in shape and position in different families. From each a vas efferens arises, and these unite to form a vas deferens. This discharges at the genital pore, situated usually ventrally, near the bifurcation of the gut in front of the ventral sucker. The terminal part is often modified to form a muscular copulatory organ, the cirrus. There is a single ovary from which the oviduct arises. This gives off a branch which ends blindly in a receptaculum seminis, but which may give off a secondary branch (Laurer's canal) which opens externally on the dorsal surface and is believed to take care of the overflow from the oviduct. The oviduct proper, in which fertilization of the ova takes place, receives ducts from the yolk glands and the shell gland, and continues forward as the uterus. The uterus discharges the ova at the common genital pore. The terminal part functions as a vagina at the beginning of sexual activity, but after the uterus becomes filled with ova, copulation seems impossible.

Classification.—Flukes are divided into two subclasses: (r) the Monogenea, in which the egg gives rise to a larva which becomes an adult without an intermediate host; and (2) the Digenea, in which the larva first becomes parasitic in some intermediate (molluscan) host, and there gives rise to subsequent generations of larvae of which some final stage develops into adults in the vertebrate host, either directly or after having evolved further in one or more subsequent intermediate hosts. The flukes parasitic in man belong to the latter subclass.

The three important superfamilies of flukes parasitic for man are:

- 1. Paramphistomoidea—flukes with two suckers, one situated at each extremity. This includes the genera Gastrodiscus and Watsonius.
- 2. Fascioloidea—flukes with two suckers, one terminal, the other posterior to it and situated ventrally. This family includes the important genera Fasciola, Opisthorchis, Dicrocoelium, Fasciolopsis, Paragonimus, Clonorchis, Heterophyes, Metagonimus and Echinostoma.

3. Schistosomatoidea—flukes in which a leaf-like male, by an infolding of its sides, makes a channel for the thread-like female. The sexes are separate, not united in a hermaphroditic worm as they are in the Fascioloidea and Paramphistomoidea.

Key to the Important Trematodes Parasitic in $\mathrm{M}_{\Lambda\mathrm{N}}$
Superfamily Fascioloidea. Hermaphroditic. Eggs Operculated.
Genital pore posterior to acetabulum
Genital pore anterior to acetabulum
1. Flukes minute, with a third (genital) sucker
Flukes medium in size, two suckers, eggs in sputum and faecesParagonimus
2. Intestine dendritic
Intestine not dendritic
3. Testes anterior to uterus
Testes posterior to uterus 4
4. Testes branched
Testes lobed or entire
5. Flukes about 4 to 7 cm. long, eggs 150 $\mu$
Flukes about 1 to 2 cm. long, eggs 30 $\mu$
6. Flukes about 1 cm., no ring of spines, eggs $30\mu$
Flukes 2 mm., no ring of spines, eggs 30µ
Trained 2 mini, no ring of opinios, oggo Jopinio.

Life History.—In the process of development from ovum to adult fluke the organism passes through four larval stages (only three in the Schistosomatoidea). The eggs as passed in the faeces are composite; i.e., the ovum proper is surrounded by volk cells necessary for its nutrition during development. In some species segmentation is well advanced when the eggs are passed. In others it does not begin until the eggs reach water, and then, if the temperature is favorable and oxygen abundant, segmentation occurs. This results after 3 to 6 weeks in the development of a miracidium, a minute ciliated embryo without an alimentary canal and with only a primitive body space, but provided with eye spots, excretory "flame cells" and secretory cells, or, e.g., in the sheep liver fluke, with a solid organ, the proboscis, at its anterior extremity. It escapes from the shell either by rupturing it or by forcing open the operculum. It then swims about actively in the water but within 24 hours it must find a suitable intermediate host which is always a mollusc (snail). It attacks the tentacles of the snail, and by means of a lytic substance formed by the secretory cells it penetrates into the tissues and makes its way to the lymph spaces around the digestive gland at the inner tip of the shell. Here it loses its cilia and develops into a sac-like structure, the sporocyst. The latter enlarges, and the cells in the sporocyst wall proliferate to form many discrete masses which develop into rediae, the third larval stage. The rediae are eventually liberated by rupture of the sporocyst and make their way into the liver of the snail, where they undergo further development. The young rediae are minute, but they may attain a length of 2 mm. in the case of the liver fluke. They are also elongated, sac-like structures, but are characterized by possessing a simple blind alimentary canal and a collar-like constriction near the anterior extremity. Within the body of the rediae for a time a second generation of young rediae may develop and escape through a birth pore behind the collar. Eventually there develop within the body of the rediae numerous cercariae, the fourth larval stage. These are minute worms resembling the adults in having a leaf-like body provided with two suckers, and a bifurcated intestinal canal shaped like a two-tined fork. They differ in having a long slender tail which in the Schistosomatoidea

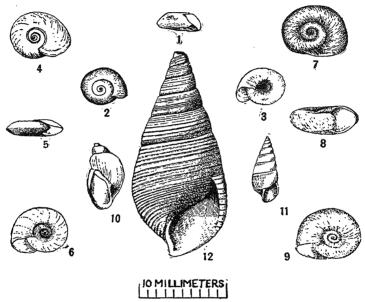


FIG. 98.—Mollusk hosts of human trematodes. 1-3, Segmentina; 4-6, Hippeutis; 7-Planorbis boissyi Potiez and Michaud; 10, Bulinus contorius; 11, Katayama noso-phora A, Adams; 12, Melania libertina Gould. (By Courtesy of Paul Bartsch.)

is forked. The visceral mass of the snail in the course of a few weeks becomes densely packed with these larvae which are continually discharged throughout the life of the snail. Faust (1934), working with S. mansoni in Puerto Rico, found that a snail infected with a single miracidium might liberate 100,000 to 200,000 cercariae.

The cercariae leave the mollusc and swim about in the water. In the case of the liver fluke they lose their tails, crawl up blades of grass, and become encysted or encyst on the water. Here they remain until the grass is eaten or the water is drunk by a sheep or other suitable definitive host. In the case of the schistosomes the free-living cercariae penetrate the unbroken skin or buccal mucous membranes. The cercariae of many flukes must quickly find a suitable second intermediate host, which is either another species of mollusc, a crab (or other arthropod), or a fish. No multiplication of the

parasite occurs in this host, which serves mainly to protect and transport the parasite. The cercariae, after entering such a host, lose their tails, penetrate into the tissues and encyst. They may undergo further growth and development as in *Paragonimus*. They then lie dormant until the host is eaten by a suitable definitive host.

Mollusc hosts.—In those trematode infections of man, of which we know the life history, some genus of mollusc serves as an intermediate host. The phylum Mollusca includes unsegmented animals, usually contained in a calcareous shell and made up of visceral mass, head, mantle and foot. The mollusc hosts of trematodes belong to the class, Gastropoda, which are known as snails and usually have a spirally coiled shell, a distinct head and a broad flat foot. The molluscs concerned in transmitting fluke discases have a foot flattened ventrally and are creeping forms (Platypoda). Gastropods with a fin-shaped foot are free swimming forms (Heteropoda). The following are the more important flukes of man with some of the genera of mollusc hosts which have been demonstrated or suspected.

There is still much difference of opinion and confusion as to the nomenclature of these snails. F. hepatica—Lymnaea truncatula; F. buski—Segmentina and Planorbis (Hippeutis); Paragonimus—Melania (and Pomatiopsis in Michigan); O. felineus—Dreissena; C. sinensis—Bythinia and Parafossarulus; M. yokogawai—Melania; D. lanceatum—Planorbis; S. haematobium—Bulinus (chiefly), Physopsis, Isodora, Lymnaea Planorbis pfeifferi; S. mansoni—Planorbis (chiefly), Physopsis, Bulinus africanus; S. japonicum—Oncomelania, Katayama (Blanfordia) and Schistosomorphora.

Lymnava, host of F. hepatica, is a common snail of ponds. It has a delicate fragile elongated shell with a pointed spire.

Melania, the host of Paragonimus, an operculated aquatic snail, has a turreted shell with an acute apex.

Planorbis, the principal host of S. mansoni, also aquatic, has a rather thick shell rolled in a flat spiral. The spire of the shell is in one plane. The shells of Physopsis, of Segmentina (the host of F. buski), and of Hippeutis have a similar shape.

Bulinus, the principal host of S. hacmatobium, has a short spire with a pointed apex and a sinistral opening. It is found in canals and ponds.

Oncomelania (with ribbed shells) and Katayama (or Blanfordia) (with smooth shells), the hosts of S. japonicum, have slender tapering shells 5 to 12 mm. long, with many whorls and a pointed apex. There is a coarse ridge on the external surface of the sharp outer lip. They are amphibious.

Experimental infection of snails by miracidia show that these tend to avoid unsuitable hosts and attack only certain hosts. Many miracidia, however, attack snails which are not efficient hosts and undergo partial development. Various species of a genus (but not all) may act as hosts. Mollusc hosts are not so restricted in their range of parasites or the flukes so restricted in their range of hosts as they were formerly thought to be.

#### LIVER FLUKES

Fasciola hepatica (Distomum hepaticum) is normally parasitic in sheep and other herbivorous animals, in which it causes the destructive disease "liver rot." It has been reported in man in over 50 cases (in Europe, South America, China, and Hawaii). The symptoms reported in most cases were mild, but it causes thickening and cystic dilatation of the bile ducts, which may lead to portal cirrhosis with cachexia and anaemia.

It is the cause of "halzoun," an affection which follows the eating of raw goats' liver. The flukes attach themselves to the buccal mucous membrane and wander into the larvnx, causing asphyxial attacks.

The fluke is about 25 mm. long and 10 mm. wide. Both intestine and testes are branched. The diameter of the oral sucker is 1 mm., of the acetabulum 1.6 mm. The eggs are ovoid, operculated, 140 by  $80\mu$ , and are deposited unsegmented. The intermediate host is a snail of the genus Lymnaea. The life history has been described.

Dicrocoelium dendriticum (D. lanceatum), also a parasite of sheep and cattle, has been reported in man in Europe and in Turkestan. The fluke, about 8 mm. long, is characterized by having the testes anterior to the uterus. It causes no symptoms of importance, and is regarded by some as an accidental parasite

Clonorchis sinensis (C. endemicus, Opisthorchis sinensis) inhabits the bile passages (and occasionally the pancreatic ducts) of man and several other mammals, including



Fig. 99.—Clonorchis sinensis. (Jeffreys and Maxwell.)

the cat, dog, pig, rat, and mouse. It is common in eastern India, China and Japan, in certain sections of which over 75% of the population are said to be infected. The fluke is 10 to 20 mm. long, 2 to 5 mm. broad, pointed anteriorly. When squeezed out of the thickened bile ducts it is so transparent and glairy as almost to resemble mucus. Many thousands of these parasites have been found in a single case. In mild infections

they may cause only minor digestive disturbances, but in severe ones, diarrhoea, enlargement of the liver, jaundice, and eventually cirrhosis and fatal cachexia.

The diagnosis is made by finding the ova in the faeces; oval yellowish brown structures  $30\mu$  by  $15\mu$ , which contain a ciliated miracidium when passed. This is not liberated until the egg is ingested by the snail. Eggs may be numerous in bile obtained through a duodenal tube even though sparse in the faeces.

The first intermediate host is a snail (Bithynia, Parafossarulus). The second intermediate host is a fresh water fish (many species of Cyprinidae). Kobayashi has shown that in cats the young flukes reach the bile ducts 15 hours after ingestion of infected fish, and require 26 days to reach maturity. Thorough cooking of the fish is essential for protection, as the cysts can survive heating to 50° to 70°C. for 15 minutes. The adult flukes have been reported to live for 5 to 20 years.

Opisthorchis felineus is normally a parasite in the bile passages of cats, dogs, and pigs. It has been reported in man in Siberia, eastern Germany, and in the Philippines, where it is fairly common. It usually causes only mild symptoms. The fluke is about 10 mm. long and 2 mm. broad. The testes are two-lobed and not dendritic. The ova are yellowish brown, about  $30\mu$  by  $12\mu$ . The first intermediate host is a mollusc, Dreissena polymorpha. Various fresh-water fish (Cyprenus, Barbus, etc.) serve as second intermediate hosts.

Opisthorchis noverca, a parasite of the dog, was found in the bile ducts of two natives in Calcutta. It was lance-shaped and covered with spines. It measures 10 by 2.5 mm., and the eggs 34 by  $21\mu$ .

#### INTESTINAL FLUKES

Fasciolopsis buski (Distomum crassum), normally a parasite of the pig, occurs in man quite frequently in India, China and Cochin China. It inhabits the small intes-

tine, and if numerous may cause diarrhoea, anaemia, and even anasarca and fatal cachexia. It is the largest trematode found in man, 40 mm. to 70 mm. long and 12 mm. broad. It is thick, brown in color, and has a very large acetabulum three to four times the size of the oral sucker and located almost adjacent to it. The branched ovary and shell gland lie in the center, with the dichotomously branched testes posterior. The coiled uterus is anterior to the testes. This species is characterized by a very long prominent cirrus. The eggs measure 80 by 130 $\mu$ , are nearly colorless and have a thin shell and a small operculum. They are passed unsegmented. From two to three weeks are required for the development of the miracidium and about seven weeks more for the development of the cercariae in the snail (various species of *Planorbis* and *Segmentina*). The cercariae require no second intermediate host but encyst on various fresh water plants, especially the water chestnut, *Eliocharis tuberosa*, and the red caltrop, *Trapa natans*, which convey the infection to man if eaten raw.

Heterophyes heterophyes (Colylogonimus heterophyes), normally a parasite of dogs and cats, occurs frequently in man in Egypt, Palestine, China and Japan. It inhabits the ileum, often in large numbers, and may cause diarrhoea. It is very small, 1.5 by 0.5 mm. It can be recognized by the large, prominent acetabulum. The oral sucker is much smaller. Very characteristic of the genus is the large, sucker-like genital pore, just below and to one side of the acetabulum and surrounded by a collar of spines. The elliptical testes lie at the extreme posterior end. The cuticle has scale-like spines. The eggs are light brown, 30 by  $17\mu$ , have a thick shell and contain a developed miracidium when deposited. Man acquires the infection by eating raw mullet, Mugil cephalus. Khalil has reported that the snail Pirenella conica serves as intermediate host in Egypt.

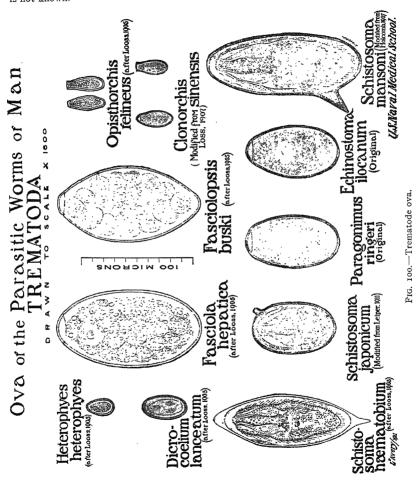
Africa et al. (1035) have reported finding several other species of heterophyids (apparently not well adapted to man as a host) in the wall of the intestine in 9 of 108 autopsics in Manila. No eggs were found in the faeces, and the intestinal lesions were trivial. However, many eggs (10 to  $16\mu$  by 25 to  $33\mu$ ) were present in the myocardium (and brain and presumably in other tissues), in which they caused capillary embolism, areas of haemorrhage, oedema and acute dilatation of the heart.

Metagonimus yokogawai (Loxotrema ovatum) is common in Japan, Korea, Formosa, Palestine and the Balkans. It is found in cats, dogs and pigs as well as man. It causes very few symptoms. It is a minute fluke r to 2 mm. long and 0.6 mm. wide, inhabiting the duodenum. The acetabulum is displaced to one side, together with the common genital pore. The latter is not surrounded by a collar of spines. The ova are about 3,3 by 20µ. The intermediate hosts are: (r) snails of the genus Melania and (2) the fresh-water fish Plecoglossus altivelus. The cercariae encyst under the scales.

Echinostoma ilocanum (Euparyphium ilocanum, Fascioletta ilocana) has been reported in a few cases from the Philippines (northern Luzon, where it is acquired by eating raw snails, Pila luzonica, etc.). It is a small fluke about 6 mm. long. There is a ring of spines around the anterior extremity. The acetabulum is prominent, about 0.5 mm. in diameter. There are two massive testes in the posterior half of the body. The eggs are 100µ long. E. malayanum, 12 by 3 mm., has been reported in man in Tamils, in the Malay States.

Gastrodiscus hominis  $(Amphistomum\ hominis)$ , normally a parasite of the pig, has been reported in man in India, China and the Malay States. It occurs in the caecum and large intestine and may cause diarrhoea. The fluke is about 6 mm. long. It consists of a posterior, concave, disc-like portion from which proceeds a teat-like projection bearing an oral sucker. The acetabulum is in the posterior margin of the disc.

The overy is behind the testes. The operculated over are 150 by  $72\mu$ . The life history is not known.



Watsonius watsoni ( $Amphistomum\ watsoni$ ) has been reported in a single case from Lake Chad, in which it caused a watery diarrhoea. It is oval, 8 mm. long, with an indistinct oral sucker and a large sucker at the posterior end. The eggs measure 130 by  $75\mu$ .

## TROGLOTREMATIDAE-LUNG FLUKES

Paragonimus ringeri, P. westermani, and P. compactus are closely related, almost identical flukes which occur as parasites in the lungs of man. They also occur in pigs, dogs, cats, and various wild carnivores. They are reddish brown in color and rather flesh-like in appearance. They are from 8 to 20 mm. long and 5 to 9 mm. wide, oval, and almost round on cross section, with, however, some flattening of the ventral surface. The acetabulum is conspicuous and lies just anterior to the middle of the ventral surface, just in front of the genital pore. The testes and ovaries are branched. The ovary and uterus are placed on opposite sides of the body, in front of the testes. The cuticle is covered with scale-like spines. The species are differentiated largely on the arrangement of the spines. In P. ringeri they are wedge-shaped and grouped in clusters of 3

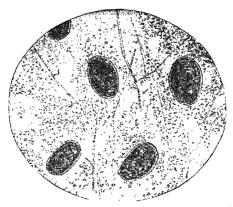


Fig. 101.—Sputum of man containing eggs of the lung fluke, greatly enlarged. (After Manson.)

to 12. In P. westermani they are scattered. In P. compactus they are clumped, but they are fewer in number and pointed. The eggs are dark brown, 90 by  $55\mu$  in size. The shell shows a thickening opposite the operculum which is most marked in P. ringers. They are unsegmented when deposited and require about 4 weeks under favorable conditions for the development of the miracidium outside the body.

The first intermediate host is a snail (Melania) (period of development 10 to 12 weeks) and the second intermediate host a crab (Polamon, Eriocheir, etc.) or in Korea a crayfish in which they undergo growth and development for six weeks more. In Japan and China these crustaceans are often eaten raw (soaked in spiced wine), but in Korea and Formosa, where the infection is also common, this custom does not prevail, and some believe that the encysted cercariae may enter the body in some other way, perhaps in contaminated water in which they may live for some time after escape from the second intermediate host.

After liberation from the ingested cyst the young fluke is believed to penetrate the intestinal wall, migrate through the peritoneal cavity, and burrow through the dia-

phragm into the lung. Here they are found, often in large numbers, in tunnel-like cavities or cysts lined by fibrous walls, which communicate with the bronchi, giving rise to many of the pathological and clinical features of bronchiectasis. Here they may live at least six years, possibly much longer. Less often the flukes invade other tissues,





FIG. 102.—Paragonimus ringeri natural size; at left showing ventral surface; at right showing dorsal surface. (Braun after Katsurada.) (From Tyson.)

including the liver, intestinal wall (when ova may be found in the faeces), testis, prostate, lymph nodes, skin, muscle, and brain. Clinically, in pulmonary infestations, there is a chronic cough with abundant, rusty brown, viscid sputum, occasional haemoptyses, wasting and loss of strength. In other sites the flukes cause various other symptoms, notably a form of Jacksonian epilepsy when present in the brain. The diagnosis is made by examination of the sputum, which contains altered blood, many ova, and usually eosinophiles and Charcot-Leyden crystals. The faeces should also be examined. The blood is said usually not to show an eosinophilia.

The infection is common in China, Formosa and Japan, where in certain districts 10% of the natives may be infected. It is common in the Philippines and occurs in India and the Malay States. A related species, *P. kellicotti*, occurs in hogs, dogs and cats in the United States and South America, but as yet only one case of human infection has been reported.

## BLOOD FLUKES

The most important of the flukes which are parasitic in man are those which are found in the blood vessels. Such infections are exceedingly common in Egypt and in certain areas in the Orient, and occur in many parts of tropical Africa and South America. The disease is commonly called schistosomiasis, since the human parasites all belong to a common genus, *Schistosoma*.

Morphology.—The schistosomes differ from other flukes in many respects. They are digenetic (sexes separate), instead of hermaphroditic. There is no pharynx. The gut-branches reunite to form a single caecum. The eggs do not have an operculum. In the larval stages asexual multiplication occurs only in the sporocyst stage, no rediae being formed. The cercariae have forked tails, but no pharynx. They penetrate the unbroken skin or mucous membranes by means of a lytic substance secreted by the single-celled cephalic glands, and do not require a second intermediate host.

The males are about 12 mm. long. The anterior fifth of the body is cylindrical, but the posterior four-fifths is flattened and thin. The margins, however, are infolded ventrally to form the gynaecophoric canal in which the female is enclosed after sexual maturity is reached. The small testes are grouped just behind the ventral sucker. The female is about 20 to 24 mm. long, filariform, and darker in color than the male. Both extremities project from the canal of the male, in which she lives. The ovary lies anterior to the union of the gut branches.

Life History.—The flukes live in the portal vein and its branches. According to the observations of Fairley and of Manson-Bahr on living monkeys infected with S. haematobium, the paired worms travel against the blood stream into the finer branches of the mesenteric or pelvic veins. The female leaves the male and penetrates as far as possible into the venules, greatly distending them. As she withdraws she deposits the ova, one at a time, with the spine pointing backwards. The elastic veins contract down about the ova, and the force of the blood stream tends to force the spine through the vessel wall. The ova cause a local inflammatory reaction and finally ulceration, so that many of them reach the lumen of the bladder or rectum, and then are passed in the urine or faeces. The eggs contain a developed miracidium when passed. On reaching water the shell quickly ruptures, probably as a result of high osmotic pressure in the egg. The miracidium escapes and seeks a suitable intermediate host. The eggs die in a few days in undiluted faeces or urine. As compared with other flukes, schistosomes produce relatively few eggs.

Infection is usually acquired by wading or bathing in infected water. Penetration of the skin by the cercariae may give rise to an intense pruritus and erythema, called "kabure," or in Puerto Rico, "piquina." The parasites (0.100 to 0.200 mm. long) burrow into a vessel, are carried by the blood stream to the lungs, and make their way to the liver and portal veins. About a month after the parasites enter the body there is often a period of fever, associated with urticarial eruptions, frequently cough, abdominal pain, and a leukocytosis with a marked eosinophilia.

The period required for the development of the characteristic local lesions varies from 6 or 8 weeks to 2 years or more. These lesions are dependent on the reaction of the tissues to the irritation caused by the eggs of the parasites. They differ in detail with the species of parasite concerned. They involve primarily the wall of the bladder or colon, in which most of the eggs are deposited. Many eggs, however, are washed out of the veins in which they were deposited and carried by the portal vein to the liver, and in smaller numbers to other organs, including the kidneys, lungs and brain. In such locations, particularly, the damage is manifestly cumulative, and not rarely leads eventually to a fatal portal cirrhosis. As suitable treatment (antimony, emetine) usually kills the adult parasites, it is evident that early diagnosis is of the utmost importance to the patient.

There are five species which have been reported in man, of which three are of major importance.

Schistosoma haematobium (Bilharzia haematobia) is the cause of endemic haematuria of Egypt, where 60% to 70% of the population is said to be infected. It is widely distributed in Africa, especially on the Mediterranean and the east coasts, in the Union of South Africa and in the Congo and Niger River regions. The male has a finely tuberculated cuticle and four testes. In the female the ovary is in the posterior half of the body. The gut-branches reunite in about the center of the body. The eggs are about 150 by  $60\mu$  and have a large terminal spine. They are deposited in the veins of the bladder chiefly, occasionally a few in the rectum. They give rise to a cystitis with haematuria, anaemia, and in chronic cases also to calculus formation, and the development of papil-

lomata which may become malignant. In many cases the infection is practically symptomless. Portal cirrhosis and splenomegaly are exceptional. The parasites are long-lived, and the disease may last 20 or 30 years. The diagnosis is made by finding the eggs in the urine, especially in the last few drops of bloody urine passed at the end of micturition, or less often in the faeces.

The intermediate host is a snail, various species of Bulinus and, in South Africa, Planorbis pfeifferi and Lymnaea natalensis.

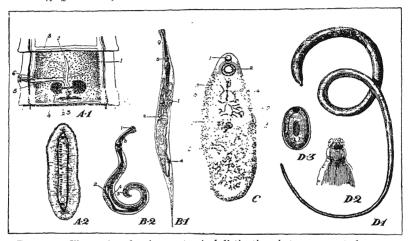


FIG. 103.—Illustration showing anatomical distinctions between a cestode, a nematode, a trematode and one of the Acanthocephala. AI. Taenia saginata; I. Testes; 2. Yolk glands; 3. Shell glands; 4. Ovaries; 5. Vagina; 6. Vas deferens; 7. Uterus; A2. Cross section of same. BI. Female Enterobius; 1. Vulva; 2. Ovary; 3. Bulb oesophagus; 4. Anus; B2. Male Enterobius; 1. Oesophageal inflation. C. Fasciolopsis buski. 1. Oral sucker; 2. Acetabulum; 3. Uterus; 4. Cirrus pouch; 5. Intestines; 6. Yolk glands; 7. Ovary; 8. Shell gland; 9. Testes. D. 1, 2, 3. Worm, head and egg of Macracanthorhynchus hirudinaceus.

Schistosoma mansoni is the cause of a chronic dysentery and of Egyptian splenomegaly. It occurs in Africa, chiefly in Egypt and in the valleys of the upper Nile, the Congo and the Niger rivers; also along the north-east coast of South America, in the Amazon valley and in the West Indies including Puerto Rico, presumably introduced by African slaves. In Africa the distribution overlaps but is not identical with that of the preceding species, depending upon the distribution of their respective intermediate hosts. The male has a more coarsely tuberculated cuticle than S. haematobium, and has about 8 testes. In the female the ovary is in the anterior half of the body, and the uterus is comparatively short and usually contains only one egg at a time. The gutbranches reunite in the anterior half of the body. The eggs are 150 by 60μ and have a

large lateral spine. They are deposited in the veins of the colon, and especially of the rectum, occasionally a few in the bladder. In their passage through the rectal wall they cause ulceration, and often extensive polyp formation, sometimes forming palpable tumors, associated with dysenteric symptoms. Many ova are carried to the liver, and

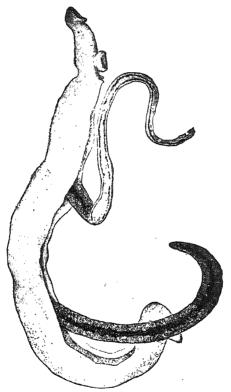


FIG. 104.—Schistosoma japonicum (male and female). The sharp edges of the borders at the beginning of the gynaecophoric canal formed by the male are an accidental appearance. (From Mense.)

cause enlargement of the liver and later a portal "pipe-stem" cirrhosis, with secondary enlargement of the spleen which may become huge. Severe anaemia is common. The diagnosis depends upon finding the *ova* in the blood-stained mucus in the faeces, or rarely in the urine. The intermediate host is *Planorbis* (various species), not *Bulinus*.

Schistosoma japonicum causes a chronic dysentery, great enlargement of the liver and spleen, anaemia, and a terminal cirrhosis of the liver. It is common in certain

districts in China, especially the Yangtse valley, in which ten million people are said to be infected, and in southern Japan, and occurs in the southern Philippines. It occurs naturally also in dogs, cats, rats, field mice, cattle, horses, and pigs.

The male has a smooth cuticle, its sides infold more markedly than do those of the preceding species, and there are 6 to 8 testes. In the female the ovary is in the middle of the body. In both sexes the gut-branches reunite in the posterior fourth of the body. The eggs are oval, transparent, measure about 80 by  $60\mu$ , and have a smooth shell but usually show a rudimentary lateral spine or knob near one end. They are excreted only in the faeces. Infection with this parasite (Katayama disease) is more serious than with the other species. There is a relatively greater accumulation of ova in the liver (and other organs), often leading to death from cirrhosis within 3 to 5 years.

The intermediate host is a snail, various species of Oncomelania, Katayama and Schistosomo phora, according to Bartsch.

Diagnosis of infection with both S. japonicum and S. mansoni depends upon finding the ova in the faeces, preferably by examining adherent mucus. The solid faeces may be well emulsified, strained, and sedimented, and the sediment examined for ova. Flotation methods are unsatisfactory. Fülleborn suggested washing the sediment 2 or 3 times with 2.5% salt solution, and each time allowing the eggs to sediment 5 minutes in the dark and decanting the fluid. Then add distilled water and expose to the light at a temperature of 120°F. The miracidia hatch quickly and by using a hand lens may be seen swimming about near the surface. Those of S. haematobium do not rise to the surface, however.

A complement fixation test has been devised by Fairley (1917), with an alcoholic extract of the livers of snails infected with S. mansoni as antigen. He obtained positive reactions in a large proportion of cases of early infection with both S. mansoni and S. haematobium, and believes it can be used to check the results of treatment. The method promises to be of great practical value, if a standardized antigen can be made available.

Fairley obtained positive *cutaneous reactions* (also group reactions) by using filtered saline extracts of infected livers. These reactions persisted after apparent cure.

Schistosoma intercalatum was described by Fisher in the upper Congo. It is almost indistinguishable from S. haematobium. The ova were relatively longer and more slender and were found only in the faeces. It causes only mild symptoms. The intermediate host is Bullinus africanus. S. bovis, a parasite of cattle, has been reported in man in a few cases from South Africa.

Prophylaxis.—Egg-containing material should be treated to kill the eggs, or it should be prevented from gaining access to water containing snails. The cercariae can live only 24 to 30 hours in water; hence, filtering or impounding infected water makes it safe for bathing or drinking, as does chlorination. The snails withstand drying, but are killed by a high dilution (1-100,000 to 1-1,000,000) of copper sulphate, which has been recommended. The organic matter in water, however, fixes the copper and renders it inert. Subsoil drainage and the pumping of live steam into the water to kill the snails appear to be the most promising procedures.

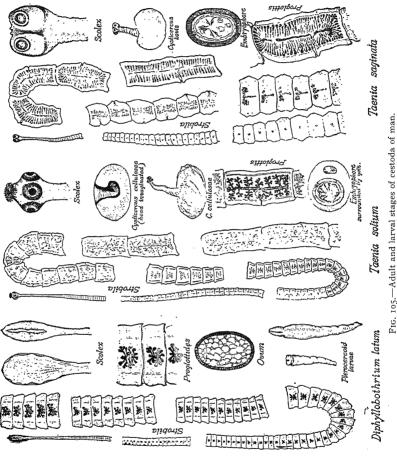
## CESTODE OR TAPE-WORM INFECTIONS

Anatomically a tape-worm may be considered as a series of individual flukes united in one ribbon-like colony. They are made up of a relatively minute "head," the ancestral or mother segment, and a series of daughter segments (proglottides) which arise from the head by a continuous process of cell proliferation. The head and the adjacent slender portion of the body in which segmentation is not distinct (the "neck") together constitute the scolex. The scolex and the proglottides constitute the strobila. The head is provided with sucking discs, and frequently with hooklets carried by a protrusible structure, the rostellum, which enable the parasite to attach itself firmly to the intestinal mucosa. These structures are important in classification. The head is the portion of the worm which is of primary importance. The permanent evacuation of one of these parasites is accomplished only when the head as well as the segments is expelled. Otherwise the strobila will be reformed from the head.

The *head* contains the central nervous tissue and the commencement of the water-vascular (excretory) system. There is no digestive system, nourishment being absorbed directly from the intestinal contents of the host.

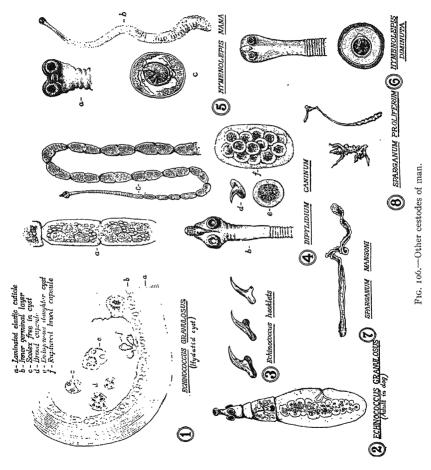
The proglottides may be regarded as sexually complete, hermaphroditic individuals, practically "egg factories." They are covered by an elastic cuticle, and in their interior contain striated elliptical bodies composed of calcium carbonate, varying from 5 to 25µ in diameter in different species. These calcareous bodies are characteristic of cestode tissue. Aside from the sex organs they contain near each lateral margin, running the entire length of the worm, a slender nerve fibre and a pair of excretory canals which usually communicate with the opposite canals by a transverse canal. The segments vary in number from 4 in the echinococcus to 3000 or more in Diphyllobothrium latum. The mature segments are at the caudal end.

Each segment contains a central uterus, often with a varying number of lateral branches, ovaries (near the ventral surface), vitelline glands for the secretion of yolk, a shell gland, and a vagina leading from the genital pore to the receptaculum seminis and to the oviduct. From the latter a duct runs to the uterus, in which the eggs accumulate. Each egg contains three pairs of hooklets. There are also minute testes, from three to many in the tapeworms of man, a system of collecting tubules and a vas deferens opening at the genital pore, and differentiated at its terminal portion into an intromittent muscular organ, the cirrus. Fertilization of ova may be effected by sperm from the same segment, or from other segments of the same worm, or of other worms. The eggs are not discharged through the genital pore. In Diphyllobothrium the eggs are discharged consecutively through a special birth pore. In Taenia there is no birth pore, the uterus ends blindly, and eggs are liberated only after disintegration of the segment. This may occur in the intestine, and then numerous eggs may be found in the faeces. In other cases one or more mature segments may detach themselves and be passed intact in the faeces (which then do not contain eggs). In the case of Taenia saginata the segment may wriggle out through the anus, or if faeces have been deposited on the ground it may creep away from the faecal mass into the grass and there disintegrate and liberate eggs in a situation in which they are likely to be eaten by a cow.



Life Cycle.—Practically all cestodes require both a definitive and an intermediate host. For nearly all species infecting man, man is the definitive host and is usually injured little if at all by the infection. The intermediate host may be another mammal, a fish or an arthropod. In the Taenioidea, when eggs are swallowed by an appropriate host, the shell is dissolved, and the liberated embryo (onchosphere) with the aid of its

hooklets burrows through the gut wall and penetrates into a suitable tissue where it encysts, producing a bladder-like structure containing fluid. The hooklets are then discarded. At one or more points the cells in the wall of the cyst proliferate and invaginate



to form a scolex. If a single onchosphere gives rise to a single cyst containing a single scolex, as in Taenia, the structure is called a *cysticercus*. If it produces a single cyst containing many scolices, it is termed a *coenurus*, as in "C. cerebralis" of sheep (the larval form of Multiceps multiceps of the dog). If it forms many cysts each containing many

scolices, an echinococcus. The term cysticercoid is applied to a cysticercus which is minute and contains very little fluid, as in Hymenolepis. When the cyst is ingested by the definitive host the scolex evaginates, attaches itself to the intestinal wall and develops into the adult worm.

Man is the intermediate host in the case of *Echinococcus granulosus*, rarely of *T. solium* and a few other cestodes, and may suffer serious injury from such infection.

#### KEY TO IMPORTANT CESTODE SPECIES FOUND IN MAN

- I. Head with two elongated slit-like suckers; genital pores ventral; rosette-shaped uterus. Bothriocephaloidea.
  - A. Single set of genital organs in each segment. Diphyllobothrium.
  - B. Double set of genital organs in each segment. Diplogonoporus.
  - C. Immature forms showing characteristics of Bothriocephaloidea (collective group). Sparganum.
- II. Head with four cup-like suckers; genital pores lateral. Taenioidea.
  - A. Uterus with median stem and a varying number of lateral branches.
    - (1) Head with two rows of hooks. Many segments. Uterus with 5 to 12 lateral branches. Taenia solium.
    - (2) Head without hooks. Many segments. Uterus with 15 to 30 lateral branches. Taevia saginata.
    - (3) Head with two rows of hooks. 3 to 5 segments only. Echinococcus granulosus.
  - B. Uterus without median stem and lateral branches.
    - (1) Genital pores single. Rostellum with not more than two rows of hooks.
      - (a) Suckers armed with numerous small hooklets. Fifteen to twenty testes in each segment. Davainea.
      - (b) Suckers not armed. Three testes in each segment. Hymenolepis.
        - Rostellum with a single row of hooklets. Length 1 to 8 cm. Hymenolepis nana.
        - (2) Rostellum unarmed. Length 20 to 60 cm. Hymenolepis diminuta.
    - (2) Genital pores double. Rostellum with four or five rows of hooks. Dipylid-

## Taenioidea Infections

Taenia saginata, the beef tape-worm or unarmed tape-worm, is found throughout the world wherever beef is eaten. Man is the definitive host, the cow the intermediate host. Next to *Hymenolepis nana* it is the commonest tape-worm in the United States.

Morphology.—The adult worm is found in the proximal part of the small intestine. It is from 4 to 8 meters long and is made up of several hundred (up to 2000) segments. The small pear-shaped head (1.5 mm.) has four pigmented elliptical suckers but no hooklets. The armed rostellum of T. solium is absent, being replaced by a depression (sucker). The mature proglottides are plump, about 18 to 20 mm. long and 5 to 7 mm. wide. There is a single unilateral genital pore which usually appears on opposite sides of adjacent segments. The uterus has 15 to 30 slender lateral branches on each side

which show forking or tree-like branching, in contrast to T. solium which shows 5 to 12 lateral divisions only. There are two ovaries.

Life cycle.—The eggs are slightly ovoid, about 25 by  $35\mu$  in diameter, and possess a thin transparent outer shell which is usually lost in the faeces. Within this there is a thick, radially striated layer (the embryophore) containing the embryo proper (the onchosphere) with three pairs of hooklets. The eggs can not be distinguished with certainty from those of T. solium.

After ingestion by the cow the onchosphere is liberated and penetrates into the tissues, especially the diaphragm, heart, tongue and masticatory muscles. Here they become encysted, forming small bladder-like structures about 6 by 8 mm. in size, containing a small scolex but relatively little fluid (Cysticercus bovis). The absence of hooklets about the head distinguishes it from Cysticercus cellulosae the larva of T. solium. They remain quiescent in the muscle but viable for many months at least. All reports of this larval stage in man are open to question.

When infected meat is eaten raw or inadequately cooked, the cyst wall is dissolved, the scolex attaches itself to the intestinal wall and grows rapidly, reaching the adult stage after about two months. The maximum duration of life is not known but is at least several years.

Prophylaxis depends upon adequately cooking beef, adequate inspection of all meat and sanitary disposal of human faeces. The parasites are easily killed by heat. They are also killed by thorough salting and by refrigeration for three weeks or more. It is probable that the various raw meat cures have made the infection more common.

Abnormalities of both the scolex and the proglottides are common. Several related species which have been described are believed by many to be merely variants of *T. saginata*. (*T. confusa*, *T. africana*, *T. philippina*, etc.).

Taenia solium, the "measly-pork" tape-worm, may be met with wherever raw pork is eaten. It is very rare in the United States, Canada and England, but is fairly common in north-east Germany and some other parts of Europe. Man is the definitive host, and the hog the normal intermediate host. However, man may serve as intermediate host of the larva.

The adult is somewhat smaller than T. saginata, rarely exceeding 1000 segments and 2 to 4 meters in length. The head is globular, 1 mm. in diameter, and has a rostellum armed with 26 to 28 hooklets. The mature proglottides are 5 to 6 mm. wide and 10 to 12 mm. long. The uterus shows 5 to 10 or 12 coarse lateral branches. There are two ovaries, but the ovary on the pore side sometimes shows a small section separated from the rest of the ovary by the vagina. The segments are passed with the faeces, but do not migrate spontaneously from the host.

After ingestion of the eggs by the hog the embryos are liberated and encyst, often in the tongue, neck and shoulder muscles, but any tissue or organ may be involved. Development takes about three months, when they attain a diameter of 6 to 12 mm. They remain viable in pork for years. Prophylaxis requires thorough cooking of pork. Smoking, pickling, and refrigeration are not sufficiently dependable.

If eggs are ingested by man or regurgitated into the stomach, a similar invasion of the tissues occurs. Any organ may be involved, including frequently the brain and occasionally the eye. Here the cysts may become quite large and cause grave disturbances. It is, therefore, very important to identify the parasite when present, to enforce rigorous precautions to avoid ingesting eggs and to institute treatment promptly.

Diagnosis of infection with worms of this genus can sometimes be made by finding eggs in the faeces, but the species can not be identified in this way. Precise diagnosis depends upon finding and examining a segment of the worm. This should be mounted between two slides and compressed sufficiently to make clear the lateral branches of the uterus. After an anthelmintic all the stool should be saved and screened and the sediment thoroughly searched for the head, using a hand lens. If the head is left behind a new worm will develop, but two or three months may elapse before eggs or segments reappear. There may be several worms present.

Hymenolepis nana ( $Taenia\ nana$ ), the dwarf tape-worm, is the smallest of the human tape-worms and the commonest in the United States. Stiles found it in 5% of the children of a Washington orphanage. It has been estimated that in parts of Italy 10% of the children are infected. It is probably identical with the common rat tape-worm, H.  $nana\ fraterna$ .

The adult worm is usually from 10 to 40 mm. long (extremes 5 to 100 mm.) and 0.6 to 0.8 mm. wide. It contains from 100 to 200 segments from 0.1 to 0.12 mm. long. It has lateral genital pores, all of which are on the same side. The head (0.3 mm.) has four suckers and a retractile rostellum encircled by a single row of 24 to 30 hooklets. The eggs are liberated by disintegration of the terminal segments. They are oval, about 35 by  $45\mu$  in diameter, colorless and translucent. There is a thin outer membrane and an inner membrane leaving a zone about  $7\mu$  wide between them. At each pole of the inner membrane there is a slight protuberance from which arise several long filaments which lie in the zone between the membranes and partly encircle the embryo. The latter contains six conspicuous hooklets.

The adults live in the upper ileum and are often numerous, 1000 to 1500 or more. They may then give rise to gastrointestinal and nervous disturbances.

Life history.—Unlike other members of this group the parasite requires no intermediate host. In the rat and probably in man the ingested eggs, after passing through the stomach into the intestine, liberate the embryos which penetrate into the villi and undergo encystment, forming a cysticercoid. After four days' development a rostellum with hooklets appears. The larva then leaves the villus, re-enters the lumen of the intestine and attaches itself to the mucosa. It develops rapidly so that after about a month eggs appear in the faeces. It is, therefore, easy for the host to become superinfected if faeces are conveyed to the mouth by dirty fingers. Contamination of food with faeces of infected rats is probably the usual source of infection.

Hymenolepis diminuta, the flavo-punctate tape-worm of rats, has been found several times in man, chiefly in children. It is 20 to 60 cm. long and about 4 mm. wide, containing about 10000 segments. The mature segments are broader than they are long. The head carries small suckers and a rostellum without hooklets. The ova resemble the preceding but are 50 to  $80\mu$ , have a thicker, radially striated, outer membrane and no filaments. The intermediate hosts are various species of insects and myriapods, including the rat flea, and infection occurs by swallowing an infected intermediate host.

Drepanidotaenia lanceolata (H. lanceolata), common in geese and ducks, has been reported once in man. It is from 2 to 12 cm. long and 6 to 12 mm. wide. It has a small globular head with a rostellum bearing 8 hooks, and a short neck.

Dipylidium caninum, the common tape-worm of cats and dogs, has been found occasionally in man, chiefly in children. It is 15 to 30 cm. long and 3 mm. in maximum breadth. The segments are shaped like melon seeds, 8 to 10 mm. long, and have bilateral genital pores. The head has four suckers and a rostellum with three or four rows of encircling hooklets. The eggs are similar to those of *H. nana* but are grouped in packets of 5 to 20 in the faeces. Infection is acquired by swallowing infected dog fleas or lice which constitute the intermediate hosts.

Davainea madagascariensis, normally a parasite of birds, has been reported in a few human cases. (Madagascar, Siam, British Guiana, Formosa, Philippines.) It is 12 to 18 inches long. The genital pores are unilateral. The head has four suckers and a rostellum with 90 hooklets. The suckers have rings of hooklets. The elongated ova are deposited several in a packet. The cockroach is thought to be the intermediate host.

Echinococcus granulosus (Taenia echinococcus) is a parasite of the dog, wolf and other carnivores, the normal definitive hosts. The cat can be infected. The adult worms live in the small intestine. They are minute, 4 to 5 mm. long. The head has four suckers and a rostellum encircled by two rows of hooks. There are only 3 (sometimes 4 or 5) segments. The terminal one is usually larger than all the rest of the worm and is packed with ova. The eggs (in the faeces of the dog, not man) closely resemble those of T. Saginata.

The usual intermediate hosts are cattle, sheep, and hogs, but man and many other mammals may also be infected. The disease occurs wherever man lives in such close association with dogs that the hands or food become contaminated with their faeces. It is common in Iceland, Arabia, and in parts of central Europe, north and south Africa, South America and Australia. It is rare in man in the United States, but is common in swine in some parts of this country.

In the digestive tract the eggs lose their membranes. The liberated embryo penetrates the intestinal wall and within eight hours passes as a rule to the liver (60%), probably through the portal vein. In some cases it is carried to other organs, the lungs, kidneys, spleen, omentum, heart, brain or to almost any organ or tissue; but as a rule only one organ is affected. The larva penetrates into the tissues and becomes encysted. The cyst develops a wall with two layers. (1) The outer layer is thick, laminated and elastic, so that it curls up when incised; (2) the inner layer is made up of a protoplasmic matrix containing numerous nuclei. Around the cyst there is also a connective tissue appeals formed by the tissues of the host. From the inner or germinal layer bud-like processes arise, which become vesicular and are termed brood capsules. By a process of localized proliferation and invagination of the wall of the brood capsules numerous scolices are produced. Each scolex is about 0.175 mm. in diameter, is borne on a pedicle

and shows suckers and two rows of hooklets. Some of the brood capsules separate from the walls and settle to the bottom of the cyst as a fine granular sediment known as "hydatid sand"; liberated scolices also may be present.

As the cyst ("hydatid") gradually enlarges. (reaching a diameter of about 10 cm. after 10 months), invaginations of the wall may give rise to secondary or daughter cysts (possessing a laminated external layer as well as a germinal layer), but such cysts usually arise by development from brood capsules or scolices. From them grand-daughter cysts may arise in a similar manner. In each there is a continuous formation

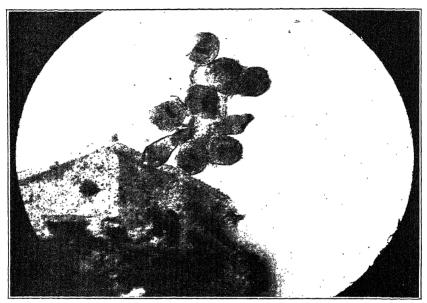


Fig. 107.—Echinococcus cyst wall with a ruptured blood capsule showing scolices. (From U. S. Naval Medical Bulletin.)

of brood capsules and of scolices in each capsule. Growth may continue for several years (2 to 8), and the cyst may become as large as a child's head (although usually they are much smaller). It has been estimated that as many as 2,000,000 scolices may thus arise from a single ovum. Occasionally a cyst is barren, contains no scolices. When the contents of a cyst are eaten by a dog, each scolex may develop into an adult worm.

In some cases in which no effective encapsulation occurs the daughter cysts develop as a result of evaginations of the cyst wall. This results in the formation of a mass of small discrete vesicles, like a bunch of minute grapes, which tend to infiltrate the surrounding tissues and even to metastasize to other organs like a malignant tumor ("Gallertkrebsen"). They occur most often in the liver, especially in cattle. Because these multilocular or alveolar hydatids are common in man in certain districts (southern

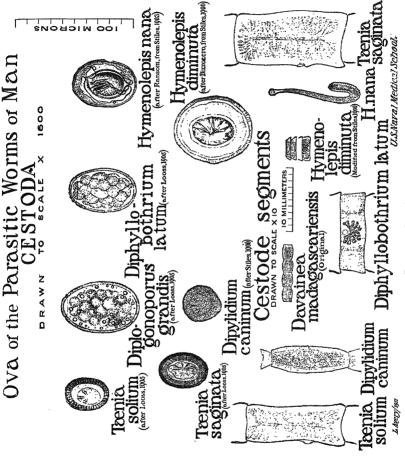


Fig. 108,—Cestode ova.

### FLAT WORMS

Germany, Russia) but are almost unknown in others (Iceland, Australia), some have regarded the parasite as a distinct species, *E. multilocularis*; the present evidence is against the idea that this is a distinct species.

Clinical Symptoms.—The cysts eventually cause grave injury as a result of pressure and destruction of the organs involved. In the liver the early stages are usually symptomless, or a tumor may be felt. Large cysts on the convex surface may be mistaken for a pleural effusion. If the contents become infected the symptoms are those of abscess. Ruptufe may occur spontaneously, either into the peritoneal cavity, pleura or lung, less often into the gastrointestinal tract or into the tissues, and usually causes a violent reaction which may be fatal. Anaemia, emaciation and weakness develop, and death usually occurs within a few years unless complete surgical removal is possible.

Involvement of the lung occurs in about 12% of the cases and is even more serious. The cysts are most often in the right lower lobe. The early symptoms, cough, small haemoptyses, bouts of fever, transient localized rales, suggest early tuberculosis. Later the symptoms and signs are those of tumor or abscess. Rupture may occur into a bronchus, the pleural cavity, or both. If such an accident is not quickly fatal, recovery may occur spontaneously or after suitable surgical drainage. The sharp outline of the cyst in roentgenograms is characteristic. Eosinophilla is usually present. Hooklets may usually be found in the sputum after rupture if search is prolonged. Early excision should be attempted if the cyst is favorably located.

Diagnosis may be made: r. By examination of fluid from a cyst obtained at operation (or autopsy). Exploratory aspiration is dangerous. If leakage of fluid occurs, or if a cyst ruptures, a violent reaction occurs, anaphylactoid in type. If scolices are scattered, they tend to become implanted and give rise to new cysts. The fluid is clear, contains about 0.5% of NaCl, a trace of sugar, no albumin. The clinical symptoms which formed the basis for supposing a "toxin" to be present are probably manifestations of an anaphylactic reaction. Diagnosis depends upon finding free scolices or scattered hooklets from disintegrated scolices. These are 25 to  $40\mu$  long, and have a characteristic shape (see Fig. 106). The appearance of a fragment of the curled laminated cyst wall is also decisive.

- 2. By the *precipitin test* of Welch and Chapman. Equal parts of patient's serum and clear hydatid cyst fluid are mixed and incubated one hour at 37°C. A positive reaction (a precipitate) is obtained frequently if the antigen is good, but false positive reactions occur.
- 3. By the complement fixation test of Weinberg and Parvu. The usual Wassermann technique is followed, using as antigen 0.4 cc. (or ½ to ¼ the anticomplementary dose) of cyst fluid. The fluid may be obtained from sheep cysts, and is filtered, carbolized and inactivated before use. The results appear to be reliable, but there is difficulty in preserving the antigen.
- 4. By cutaneous allergic tests (Casoni). A small drop of cyst fluid is injected intradermally or applied to a scratch. A positive reaction, con-

sisting in the rapid appearance of a large wheal with a zone of erythema, has been reported in about 90% of the cases. There may be a marked secondary late reaction also. Sensitiveness may last long after removal of the cyst.

If removal is attempted the cyst should be excised intact, or else the wall should be stitched to the abdominal wall and allowed to heal by granulation (marsupialization). It is customary to inject some antiseptic fluid into the cyst to kill the scolices before undertaking any manipulations which might result in accidentally scattering the contents of the cyst.

# Bothriocephaloidea Infections

Diphyllobothrium latum (Dibothriocephalus latus), the broad Russian tape-worm or fish tape-worm, is common in Scandinavia, Russia, Switzerland, Bavaria, Central Asia, Japan and central Africa. It was introduced years ago into the Great Lakes region of North America. It is harbored also by dogs, cats and bears, which become infected by eating raw fish.

Morphology.—The head is olive-shaped, 2.5 mm. in diameter and has two deep suctorial grooves (bothria) on each side but neither rostellum nor hooklets. The body attains a length of 30 feet (rarely even 60 feet) and may contain 3,000 or 4,000 segments which are about 12 mm. broad and 5 mm. long. If several worms are present, they are smaller. The uterus is rosette-shaped, and there is a ventrally placed genital pore. Each mature segment is continuously discharging eggs which are present in large numbers in the faeces (estimated at 36,000 per day). They are operculated, brownish in color, 45 by  $70\mu$ , with a thin shell enclosing a central mass of granular spherical segments.

Life history.—When the eggs reach fresh water, if the temperature is favorable, segmentation proceeds, and after three weeks the operculum opens and a ciliated embryo, the coracidium (six-hooked onchosphere), 25 to 30µ, escapes. It can live several days in water, swimming about until it is swallowed by a cyclops (C. strenuus, Diaptomus gracilis, D. oregonensis etc.), the first intermediate host. It loses its ciliated covering, pierces the gut wall, and after 15 days' development in the tissues becomes a procercoid larva, an ovoid structure 50µ long with a spherical protuberance at one end containing six hooklets. When the cyclops is eaten by certain fresh water fish (pike, pickerel, perch, trout, salmon etc.), the second intermediate host, the larva penetrates the stomach wall and passes into the muscles or other tissues. Here it develops into a plerocercoid larva or sparganum, a worm-like organism 8 to 16 mm. long with suctorial grooves at its anterior extremity. When the raw fish is eaten by man or other suitable definitive host, the larva is liberated in the small intestine and develops rapidly into the adult form. After 18 days it may reach a length of two feet and begin to discharge eggs. It may live for years, even it is said for 16 years.

Prophylaxis depends upon adequately cooking the fish. The effect of smoking, salting or pickling has not been studied adequately, but at present one can not rely on these procedures. The infection is usually symptomless. The well known macrocytic anemia is a very rare complication (in Finland about 1 or 2 in 10,000 cases).

Diphyllobothrium mansoni (D. erinacei) is an allied but much smaller species found in dogs, cats and related carnivores in Japan, China, Australia and east Africa. The

normal intermediate hosts are (1) a cyclops, and (2) either a frog or a snake. According to Manson-Bahr about 60 cases have been reported of human infection with the plerocercoid stage ("Sparganum mansoni"). These larvae resemble ribbon-like strings of fat 3 to 12 inches long and may be encapsulated in many different tissues, not rarely in the conjunctiva where they cause serious disturbances. Human infection might come from swallowing infected cyclops in contaminated drinking water. There is evidence, however, that infection may be brought about by the Chinese custom of applying split live frogs to sores on the hands or to the eyes as a dressing, the parasite migrating from the frog into the tissues of the wound.

Sparganum proliferum is a plerocercoid larval form reported in five human cases (Japan and Florida) in enormous numbers in the tissues. The adult form and life history are unknown. The larvae multiply in the tissues asexually, by forming lateral buds.

Diplogonoporus grandis, a tape-worm with two complete sets of genital organs in each segment, has been reported in six cases from Japan. It is from 1.5 to 6 meters long, and the mature segments are about 2.5 mm. broad and 0.5 mm. long. The ova are operculated, about 50 by  $65\mu$ . The life history is not known. Infection is probably acquired by eating raw fish which are believed to be the second intermediate host. According to Brumpt the usual definitive hosts are whales and certain aquatic birds.

# CHAPTER XX

# ROUND WORMS

CLASSIFICATION OF THE NEMATHELMINTHES (ROUND WORMS)

### Class Nematoda

Superfamily	Family	Genus	Species
Rhabdiasoidea	Strongyloididae \Trichuridae	Strongyloides	S. stercoralis
Trichuroidea	Trichinellidae	Trichuris	T. trichiura
	( i richinellidae	Trichinella	T. spiralis
	Ancylostomidae	Ancylostoma	A. duodenale A. braziliense
		Necator	N. americanus
		(Ternidens	T. deminutus
		Oesophagostomum	O. brumpti
Strongyloidea	Strongylidae	Ocsophagostomam	O. thomasi
		(	
	Trichostrongylidae	Trichostrongylus	T. colubriformis
		(Haemonchus	H. contortus
	Metastrongylidae	Metastrongylus	M. apri
Dioctophymoidea	Dioctophymidae	Dioctophyme	D. renale
Oxyuroidea	Oxyuridae	Enterobius	E. vermicularis
		Ascaris	A. lumbricoides
Ascaroidea	Ascaridae	Toxascaris	T. leonina
		(Toxocara	T. canis
Spiruroidea	Spiruridae	Physaloptera °	P. caucasica
		/Loa	L. loa
		Wuchereria	W. bancrofti
Filarioidea	Filariidae	Filaria	F. malayi
		Mansonella	M. ozzardi
		Acanthocheilonema	A. perstans
		Onchocerca	O. volvulus
	Dracunculidae	Dracunculus	D. medinensis
Acanthocephala	Gigantorhynchidae	Macracanthor- hynchus	M. hirudinaceus
(Class)	(Moniliformidae	Moniliformis	M. moniliformis
	Hirudinidae	∫ Hirudo	H. medicinalis
Annelida (Phylum)	THRUMINOSE	Limnatis	L. nilotica
Hirudinea (Class)	Haemadipsidae	Haemadipsa	H. zeylanica

The subphylum Nemathelminthes is divided into two classes: the Nematoda which possess a gut but are without a proboscis and the Acanthocephala in which the gut is absent but proboscis present. The former includes the subclasses Eunematoda and Gordiacea of which the latter are accidentally parasitic in man. The Eunematoda contains normally free-living forms (order Vagantia of some authors) which are occasionally introduced accidentally into man and the more important parasitic forms (order Parasita of some authors). Eight superfamilies of the parasitic forms are included in the Key from Yorke and Maplestone, but several of the groups here classed as families are regarded as superfamilies by some authorities.

Note.—The Annelida are grouped with the round worm table for convenience only and not to show taxonomic relationship. The Acanthocephala are not closely related to the nematodes, and their taxonomic position is still in doubt.

#### ROUND WORMS OR NEMATODES

All nematodes are covered with a cuticle varying in thickness and frequently ringed. Characteristically the cuticle is moulted four times during development. The cuticle is formed by the underlying ectoderm which is as a rule markedly thickened internally so as to form four ridges which divide the body into quadrants. Within the ectoderm is the body cavity, a space containing clear fluid in which the reproductive organs lie. The excretory system usually consists of two tubes which discharge near the head.

While the alimentary canal is more or less tube-like in appearance it shows near the mouth a distinct oesophagus. This may have a scanty musculature and lie apposed to a single row of large secretory cells (Trichuroidea); it may be muscular without a globular bulb (Strongyloidea) or muscular with a posterior bulb (Oxyuroidea). In Filarioidea and Spiruroidea the oesophagus may be divided tandem. There is a nerve ring around the oesophagus.

The testis and ovary are generally tube-like. The sexes are, as a rule, separate. The male can usually be recognized by its smaller size, its curved or curled posterior end, at times exhibiting an umbrella-like expansion—the copulatory bursa. The spicules, chitinous copulatory structures, may be observed drawn up in the worm or projected out of the cloaca. The genital opening of the female is ventral and may vary in position from close to the mouth to near the tail. That of the male is close to the anus, and both open into a common cloaca which opens in the ventral line as the cloacal aperture. Certain papillae in the region of the anus are valuable in differentiation of species.

Many nematodes develop in damp earth from the eggs as rhabditiform larvae. Very few nematodes are viviparous (Wuchereria, Trichinella), most of them being oviparous (Ascaris).

The parasitic Eunematoda are divided into at least eight superfamilies.

# 

- 4. Bursa copulatrix cuticular and supported by rays.......Strongyloidea

  Bursa copulatrix muscular and not supported by rays......Dioctophymoidea
- 5. Oesophagus dilated posteriorly into a bulb usually containing a denticular apparatus and frequently separated from the rest of the oesophagus by a constriction....

- 7. Usually with two lateral lips, chitinous buccal cavity or vestibule usually present, vulva usually in the middle of the body or posterior to it; parasites of alimentary canal, respiratory system, or orbital, nasal or oral cavities...........Spiruroidea Usually without lips, buccal cavity or vestibule absent or rudimentary, vulva almost invariably in the oesophageal region; parasites of the circulatory or lymphatic system, or muscular, or connective tissue, or of serous cavities..........Filarioidea

The family Anguillulidae (normally free-living nematodes occurring only accidentally in man) contains the genera *Rhabditis* and *Anguillula*. Several species of *Rhabditis* have been reported from man. *Anguillula aceti*, the vinegar eel, has been reported from the genitourinary tract several times. Such cases can be explained by the prior contamination of the urine bottle, or by the use on the part of the patient of a vinegar vaginal douche.

# RHABDIASOIDEA

This superfamily is characterized by having two heterogenetic generations, one of free-living rhabditiform males and females and one of parasitic, filariform females. Family Strongyloididae.

### Strongyloididae

Strongyloides stercoralis.—This parasite is especially common in Cochin China and Brazil, but is widely distributed in tropical and semi-tropical regions and is fairly common in the southern United States. The account of the life history which follows is based on the work of Kreis and of Faust.

The intestinal form, formerly known as Anguillula intestinalis, a female long regarded as parthenogenetic, lives deep in the mucosa of the jejunum. It is about 2.5 mm. long and 40 to  $50\mu$  wide, has a pointed four-lipped mouth and a filariform oesophagus one fourth the length of the body. The anus is near the sharp posterior end and the vulva near the posterior third of the body. The double uterus occupies the middle and posterior thirds of the body and contains a row of 8 to 10 large elliptical eggs nearly as wide as the parent worm. The worm is so translucent that it is difficult to detect it in the mucosa, even with a hand lens. The mucosa should be scraped off and the preparation searched with a  $\frac{3}{6}$  inch objective.

The ova when discharged from the vulva closely resemble hook worm ova but are strung out in a chain by a thin transparent sheath-like membrane and might possibly appear in the faeces, but only after brisk purgation. Segmentation is well advanced,

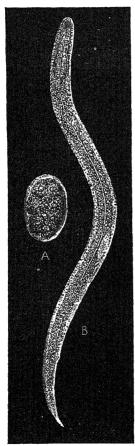


FIG. 109.—A, Egg of Strongyloides stercoralis (parasitic mother worm) found in stools of case of chronic diarrhoea; B, Rhabditiform larva from the stools. (William Sidney Thayer, in Journal of Experimental Medicine.)

however, and normally the ova quickly give rise (in the intestine) to rhabdiliform larvae about 250µ long and 13µ broad, characterized by a double oesophageal bulb. They closely resemble the corresponding stage of hookworm larvae which may hatch in incubated stools, but the depth of the mouth cavity anterior to the oesophagus is only one third the width of the larva, while in the hookworm larva it is about equal to the width. The rudimentary genital organ is conspicuous and about 20µ long, while in the hookworm it is small and inconspicuous (see Fig. 112). Usually fresh faeces contain larvae in strongyloid infection but ova in hookworm infection.

The larvae grow rapidly and at suitable temperatures (over 25°C.) develop in 3 to 5 days into free-living males and females. Both retain the rhabditiform oesophagus. The male is about 750µ long and 40 to 50 m wide and has an incurved tail and two spicules. The female is about 1 mm. long, 50 to 60µ wide, with an attenuated tail and a double uterus containing several ova. Following copulation (in the faeces outside the body) the ova are discharged and quickly give rise to rhabditiform larvae identical in appearance with those of the preceding generation hatched in the intestine. These develop in 3 or 4 days into filuriform larvae about 0.6 mm. long, with a simple tubular oesophagus, the infective stage. ("Indirect" or heterogenetic cycle.) These are distinguished from the corresponding stage of hookworm larvae by the length of the oesophagus which, in Strongyloides, is one half instead of only one quarter of the length of the body. See Fig. 110.

If the temperature is below 25°C. or as a result of other conditions not yet understood the rhabditiform larvae (arising in the intestine from the parasitic female) develop in the faeces directly into infective filariform larvae, skipping the sexual stage. ("Direct" cycle.) Faust has suggested that larvae from fertilized ova develop indirectly, while those from unfertilized females develop directly.

It has recently been claimed that under certain conditions some of the rhabditiform larvae may develop in the intestinal contents into infective filariform larvae with-out the usual period of growth outside the body. ("Hyperinfective" cycle.) These are said to penetrate the intestinal wall and immediately go through the usual developmental cycle, superinfecting the host.

Infection of man may take place by ingestion of the larvae which then penetrate the mucous membranes, or the larvae may penetrate directly through the skin. They then pass through the blood stream to the lungs where they remain for some days, developing according to Faust into adolescent males and females. Here they cause more or less acute inflammation, as do Ascaris larvae. Filariform larvae may be found in the sputum. According to Faust insemination occurs chiefly in the lungs. Some worms start reproduction in the lungs. Most of the parasites then pass by way of the trachea and the oesophagus into the intestine, where the female bores into the mucosa and begins to deposit ova about two weeks after penetration of the skin. The parasitic males, which are only 0.5 to 0.8 mm. long and resemble the free-living males, do not penetrate the mucosa and are quickly eliminated. After the supply of sperm is exhausted the female continues for a time to form ova parthenogenetically.

If the perineum is left soiled with infected faeces, development may take place in situ, and the filariform larvae penetrate the skin, superinfecting the host. They may produce local itching urticarial lesions, a manifestation of hypersensitiveness to strongyloid protein. Fülleborn has shown that local allergic reactions may be obtained by cutaneous application of extracts of dried larvae.

Diagnosis is made by demonstrating the (moving) larvae in the fresh faeces. If the stool is solid, a cup-shaped cavity may be scooped out of the mass, filled with water and incubated over night. The larvae migrate into the water and can be found easily.

The infection is usually symptomless but is very persistent (up to 24 years). In some cases it causes intestinal irritation and diarrhoea which may be violent in severe cases and lead to dehydration and exhaustion. Gentain violet (orally) is said to be a specific remedy.

# TRICHUROIDEA

These have a long thin neck and a thicker terminal portion. The oesophagus has a thin wall and is apposed to a single row of large secretory cells. The anus is terminal. There is only one ovary.

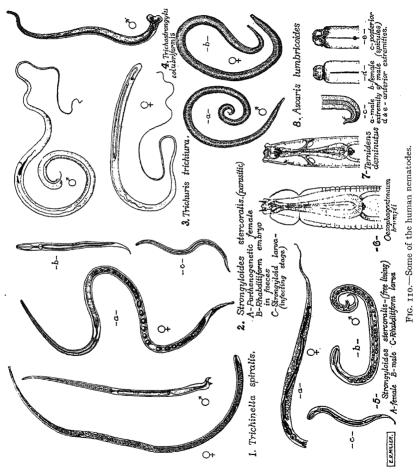
The families Trichuridae and Trichinellidae are distinguished by the latter being much smaller, not having a spicule and copulatory sheath and being viviparous.

#### Trichuridae

Trichuris trichiura (Trichocephalus dispar), the whip worm, is one of the most common parasites in both temperate and tropical climates. The worms are 30 to 50 mm. long, the females slightly longer than the males. The cephalic half to two thirds of the body is thread-like and contains only the oesophagus. This has a reduced musculature and is apposed to a row of large secretory cells, the stichocyte, which communicate with the lumen of the oesophagus by small apertures. The thick caudal portion of the body (the handle of the whip) contains the intestine and sex organs. The tail of the male is coiled and ends in a single terminal spicule surrounded by a rough sheath. The tail of the female is comma shaped. The vulva opens at the cephalic end of the thickened segment of the body. See Fig. 110.

The worms are found chiefly in the caecum, also in the appendix and terminal ileum. They attach themselves to the intestinal wall by transfixing a fold of mucosa with the slender neck and have been thought by some to facilitate the entrance of typhoid bacilli

and other pathogenic bacteria into the tissues. The characteristic ova are deposited in the faeces. They are oval structures  $22\mu$  by  $50\mu$ , brown in color, with unsegmented granular contents and a thick translucent capsule which has a knob-like protrusion at



each end, somewhat like a bottle stopper. Segmentation occurs outside the body and is a protracted process requiring from 6 weeks to (it is said) 18 months. The ova are resistant (except to desiccation) and have been reported to have retained their infectivity

for five years. There is no intermediate host. Infection occurs by swallowing infective ova. The parasite matures about one month after infection.

The infection is usually symptomless. In rare instances it has been held responsible for serious illness characterized by gastrointestinal and nervous disturbances and severe

#### Trichinellidae

Trichinella spiralis ( $Trichina\ spiralis$ ), the cause of trichinosis, is a minute worm, visible with an ordinary hand lens, which in its adult stage inhabits the duodenum and jejunum. The male is about 1.5 mm long and  $40\mu$  wide. There is a prominent testicular enlargement filling the wider caudal end of the body and two tongue-like caudal appendages which project laterally and enable the male to hold the female during copulation. There is no spicule, and the cloaca is evaginated to act as a copulatory organ.

The female is 3 to 4 mm. long and 60µ broad and has a rounded posterior extremity with a prominent slit-like cloaca. The posterior half of the body contains the ovary and becomes swollen as the eggs develop. The vulva is in the anterior fifth of the body. After copulation the males die, and the viviparous females burrow into the intestinal mucosa and for about 6 weeks continue to emit living larvae, each producing a number estimated at from 1000 to 10,000 in all. The larvae are about 100 \mu by 6\mu in size. They penetrate into lymphatics or veins and are distributed through the general circulation. They soon penetrate into striated muscle, being especially numerous in the diaphragm, intercostals, and muscles of the neck, larynx, tongue and eye. Here they become encysted larvae, forming oval structures about 0.45 by 0.25 mm. in size. They are surrounded by a tough capsule produced by the tissues of the host, while the larva. which has attained a length of I mm., is coiled up in the center. Here they remain viable for a long time (some even for 10 to 20 years) although the larva eventually dies and the cyst becomes calcified. When the cysts are swallowed the larvae are liberated in the intestine and within a few days develop into mature adults. This parasite. therefore, differs from practically all other nematodes in that the larvae encyst in the same individual host which harbors the adult worms.

Transmission.—Under natural conditions the infection chiefly affects rats and hogs. Hogs become infected by eating rats, and rats, by eating scraps of pork at abattoirs. Many other animals, however, are also susceptible. Man acquires the infection by eating raw or inadequately cooked pork. The disease occurs throughout the world where such pork is eaten. It is not uncommon in the United States and is often unrecognized. Prevention depends on adequately cooking pork. Refrigeration for a week at o°F. (-18°C.) also kills the parasites. Meat inspection is not sufficient, although in Germany it reduced the incidence of infection notably. The severity of the disease depends primarily on the number of worms swallowed. In severe epidemics the mortality often reaches 30%. Individuals who have acquired an unusually severe infection (as by eating jerked bear meat) may die quickly before an eosinophilia has developed.

Diagnosis.—During the first few days after infection, while the ingested larvae are developing, there is often a gastroenteritis. Purging then may be beneficial by ridding the intestine of the infected pork and of some of the parasites. The adults can occasionally be found (but usually are not) by repeatedly suspending the faeces in water and decanting, and examining the sediment with a hand lens.

If possible secure some of the suspected meat and examine it for encysted larvae. The meat may be teased or pressed into a thin layer between heavy glass slides and examined with the  $\frac{2}{3}$  inch objective. If no cysts are found, chop up some of the meat and digest over night at  $37^{\circ}$ C. in artificial gastric juice (pepsin in 0.3% HCl). Then put the material in the Baermann apparatus (p. 501) and after a few hours examine the fluid in the stem of the funnel for larvae by means of the microscope ( $\frac{2}{3}$  inch objective).

Instead, some meat may be fed to several white rats or mice. After 2 or 3 days examine the duodenal contents of one animal for adult worms. After two weeks examine the muscle of the diaphragm etc. for encysted larvae as above.

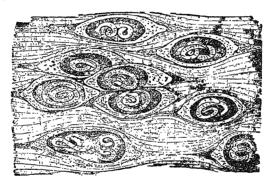


Fig. 111.—Trichinella spiralis. (Ziegler.)

During the second and third week in severe cases there is high fever, marked intoxication and prostration, as in typhoid fever. There is often transient oedema, especially about the face and eyes. There is usually a high leukocytosis with a marked eosinophilia, although this may be absent in the early stages and in fulminant cases. The larvae are then present in the circulating blood. To demonstrate them withdraw about 5 cc. of blood and lake in 3 volumes of  $3\frac{e_i}{c}$  acetic acid. Centrifugalize and examine films of the sediment stained by Giemsa's method.

From the tenth day on, the larvae begin to migrate into the muscle and become encysted. This is associated with severe pain, stiffness and disability. There is often painful labored breathing, laryngitis with cough and occasionally haemoptysis. After this time excise a bit of muscle from the deltoid or pectoral muscle near its insertion, and examine for encysted larvae, as described above.

Cutaneous reactions.—Bachman (1928) reported positive precipitin and intracutaneous reactions in infected animals, using an antigen which he obtained by digesting heavily infected muscle in pepsin and HCl and by washing, drying, grinding and extracting the larvae so liberated. Coca's solution may be used. The intradermal test has

been applied successfully to the diagnosis of human infection, especially by McCoy et al. (1933), who obtained (in Rochester, N. Y.) positive reactions (the immediate production of a large wheal) in 92% of 36 acute cases and in 60% to 80% of cases from 3 months to 7 years after infection. They gave 0.1 cc. of a 1 to 10,000 and if this is negative, a 1 to 500 dilution. Late reactions occur, but McCoy found them unreliable in human infection. In most cases the reaction became positive during the third week. In a

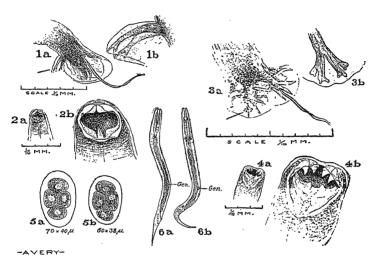


Fig. 112.—1a. Copulatory bursa of Necator americanus, showing the deep cleft dividing the branches of the dorsal ray and the bipartite tips of the branches; also showing the fusion of the spicules to terminate in a single barb. Scale \( \frac{1}{10} \) mm. rb. Branches of dorsal ray magnified. 2a. The buccal capsule of N. americanus. 2b. The same magnified. 3a. Cop. bursa of Ancylostoma duodenale, showing shallow clefts between branches of the dorsal ray and the tridigitate terminations. Spicules hair-like. 3b. The dorsal ray magnified. 4a. The buccal capsule of A. duodenale, showing the nuch larger mouth opening and the prominent hook-like ventral teeth. 4b. The same magnified. 5a. Egg of N. americanus. 5b. Egg of A. duodenale. 6a. Rhabditiform larva of Strongyloides as seen in fresh facces. 6b. Rhabditiform larva of hookworm in facces cight to twelve hours after passage of stool.

control series 18% gave positive reactions, which is practically the incidence of infection found by Quéen (1931) in a special study of 344 routine autopsies (also in Rochester. N. Y.). However, in a series of 124 cases in rural Louisiana heavily infected with other nematodes (chiefly *Trichuris*) but probably free from *Trichinella*, 62% gave positive reactions, presumably group reactions. The test promises to be of considerable practical value, but a positive result is not conclusive, particularly if other parasites are present. A positive precipitin reaction appears too late in the disease to be of practical diagnostic value.

In investigations being carried out in the National Institute of Health, under the direction of Dr. W. H. Wright, the incidence of trichina infestations in post-mortem examinations of over 800 individuals dying in hospitals in Washington and in other eastern cities was over 16 per cent. Certain population groups had an incidence approximately twice as high as that of other groups.

### STRONGYLOIDEA

In this superfamily the male has a caudal bursa, a prehensile sort of expansion at the posterior end for copulatory purposes.

The mouth is usually provided with six papillae and at times with a chitinous armature. Oesophagus of adult is without posterior globular bulb but may be greatly swollen.

Families discussed: Ancylostomidae, in which there is a well developed caudal bursa and buccal capsule; aperture of buccal capsule guarded by cutting plates or teeth. Strongylidae, with well developed caudal bursa and buccal capsule; aperture of latter guarded by a corona radiata. Trichostrongylidae, having a well developed caudal bursa but without buccal capsule or when present rudimentary. Metastrongylidae, with a poorly developed caudal bursa having atypical rays and buccal capsule absent or poorly developed.

# Ancylostomidae

The hookworm infections of man are nearly all due to two species; Ancylostoma duodenale, the "Old World species," and Necator americanus, the "New World species." In fact both are widely distributed throughout tropical and subtropical regions and are found in temperate regions in mines, tunnels or other localities in which they find warmth and moisture. The adult worms are found in the small intestine (jejunum) of man, sometimes in enormous numbers (1500 or more). They attach themselves to the mucosa from which they suck blood and cause some free bleeding. As a result of this blood loss, perhaps of secondary bacterial infection and possibly also by the secretion of a toxin, they may give rise to a severe (hypochromic) anaemia, weakness, general debility and chronic ill health. with retardation of development of infected children. Very severe infections may be quickly fatal. Many cases, however, are practically symptomless. Newcomers into an endemic area usually suffer more severely than the native population which has acquired some immunity to the parasite or to its effects. Recent studies have shown the great importance of a deficient diet in the production of the anaemia, which can often be relieved by the administration of large doses of iron without elimination of the parasites. Anthelmintics, however, should also be given. The hookworm constitutes a major public health problem in

infested regions. In some localities over 90% of the population are infected.

Ancylostoma duodenale (Dubini, 1843), originally a European species, has spread over large parts of Africa (especially Egypt) and Asia and occurs in North and South America. It has been found in the tiger and in young dogs and cats. The male is about 10 mm. long and 0.4 to 0.5 mm. wide, the female about 12 by 0.6 mm. The sexes are easily differentiated by the shape of the tail, pointed in the female, broadened in the male into an umbrella-like expansion, the copulatory bursa. The large oval mouth has four claw-like teeth on the ventral side of the buccal cavity and two knob-like teeth on the dorsal aspect. It also has a pair of ventral lancets below the four ventral teeth. The anterior part of the body is filled by the oesophageal gland which secretes a substance

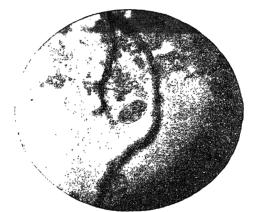


Fig. 113.—Ovum of Ancylostoma duodenale. By J. A. Thomson. (Jeffreys and Maxwell.)

inhibiting coagulation which is discharged through a dorsomedian tooth. This tooth, however, is not visible in this species. The anterior end of the body is bent rather sharply in the same direction as the general body curve. In the female the vulva is located in the posterior half of the ventral surface. The copulatory bursa of the mais shows a shallow cleft between the main divisions of the dorsal ray, which has tridigitate terminations. There are two distinct hair-like copulatory spicules. During copulation the worms join so as to form a Y shaped figure.

The females after fertilization give off great numbers of ova, variously estimated at 6000 to 20,000 or more per day for each worm. The ova measure about 40 by 60 $\mu$ . They have a thin shell, a wide clear glassy outer zone and a granular central zone which (in freshly passed faeces) is usually divided into two to four (never more than eight) segments.

Necator americanus (Stiles, 1902), the "New World species," appears to have been native in Africa and Asia and was probably brought to America by negro slaves. It is also wide spread in northern South America, the West Indies, in India, Ceylon, the

### ROUND WORMS

Malay peninsula, the Philippines and the Pacific Islands. It has been reported in some monkeys. It differs from the preceding species in the following points: It is slightly smaller, the male 8 by 0.3 mm., the female, 10 by 0.4 mm. The buccal cavity is smaller, round, and is equipped with two prominent ventral chitinous plates and two rudimentary dorsal plates in place of the teeth. The median dorsal tooth projects conspicuously into the buccal cavity. Deeper in the cavity are one pair each of dorsal and ventral lancets, or pharyngeal teeth. The anterior tip of the body is bent back sharply in a direction opposite to that of the general body curve.

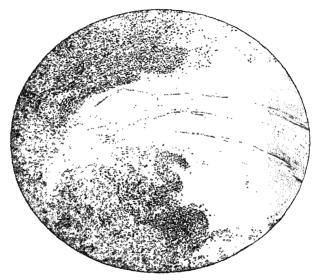
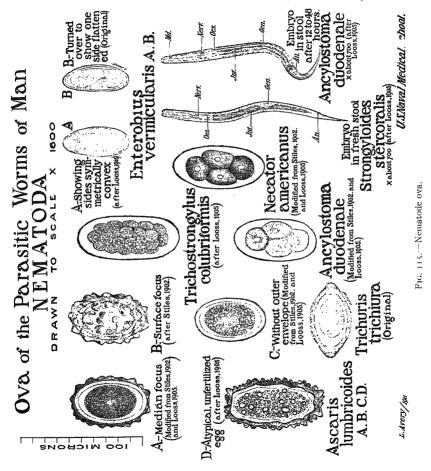


FIG. 114.—Longitudinal section through a hookworm attached to the intestinal mucosa, about six hours after the death of the carrier. A portion of the submucous coat is drawn into the buccal cavity and trails along cord-like into the eosophagus, in which 4 small tissue nuclei are still discernible. Section, o.o. mm. thick. (From Mense.)

The vulva is in the anterior half of the body. The caudal bursa is deeply cleft, and the terminal portions of the dorsal ray are bipartite instead of tripartite. The spicules are fused at their tip and end in a single barb. The ova are slightly larger (40 by  $70\mu$ ).

The life history is practically the same in both species. Segmentation of the ova does not progress within the intestine, probably because of lack of oxygen. After being passed, in the preserve of warmth (14° to 37°, preferably 27°C.), moisture (muddy water, damp earth) and oxygen, segmentation progresses rapidly so that within two days a small rhabditiform lurva is hatched. (For differentiation see p. 490 and Fig. 112.) This feeds voraciously and grows rapidly, reaching a length of 0.3 mm. on the third day,

when it moults; and 0.55 mm. on the fifth day, when it moults again, loses the bulb-like swellings of the oesophagus and is converted into a *filariform larva*, the infective stage. The old cuticle is not shed but is retained as a protecting sheath. The parasite then



enters into a resting stage in which it ceases to take food, although it remains actively motile and can crawl up blades of grass or up the vertical sides of mines. Cort found, however, that they do not migrate more than four inches from the place of development. Before becoming encysted hookworm larvace are readily destroyed by direct sunlight,

by chemicals, or even by dilution of the faeces, especially with urine. After encystment they become relatively resistant and in shaded moist ground may live four to eight weeks in the tropics (even though they lose their sheath), although according to Cort not longer than this, as some have maintained. In a cool moist environment they may live much longer. They are still quite sensitive to desiccation and to extremes of temperature.

If infected soil comes into contact with the skin, stimulated probably by the warmth, the larvae become very active and burrow through the skin causing the familiar symptoms of "ground itch." Apparently any part of the skin can be penetrated, and only a few minutes are required for the process. In the subcutaneous tissue they enter the veins or lymphatics and are carried passively to the lungs. Here they penetrate the walls of the capillaries and alveoli into the air sacs and proceed or are carried up the bronchi and trachea to the pharynx. They are then swallowed and reach the intestine about one week after infection, having undergone a third moulting. The larvae grow rapidly, undergo a fourth moulting after another week (when they measure about 2 mm. by 0.13 mm.), and reach maturity about a month after entering the body. The exact duration of life is not precisely known, but probably varies from a few months to six years. Infection can also occur if the larvae are swallowed. Fülleborn showed that in experimentally infected animals a few larvae can reach the intestine without passing through the lungs.

The number of parasites which must be present to produce disease is variously estimated and doubtless varies greatly. As a rule it probably requires 500 worms several months to cause severe symptoms. However, much depends upon the resistance of the patient. In some cases it is believed that 25 worms or even fewer may cause definite disturbances. There is not always a close parallelism between the number of worms and the severity of symptoms.

Laboratory diagnosis usually depends upon finding the ova in the faces. If numerous they can be found easily in ordinary fresh smear preparations. Examine with the low power. If sparse, concentration methods are required.

We have found Barber's technique useful. On a slide emulsify a bit of faeces in equal parts of glycerin and saturated salt solution. The ova rise to the surface and can be recognized easily with a 23 inch objective. A larger amount of faeces may be emulsified in this mixture and centrifugalized. Put a wisp of cotton on the surface of the fluid. Pour on the cotton three or four drops of melted agar. Remove the disc of agar with the cotton and examine on a slide for entangled eggs.

Pepper and independently Lane recommended the following "levitation" method. The concentrated sediment of a centrifugalized specimen is transferred to a glass slide, mixed with r cc. of water and allowed to stand 5 minutes until the ova settle on the slide. The slide (kept in a horizontal position) is then immersed in water and gently manipulated until all coarse matter has floated free. The hookworm ova stick firmly to the slide (other ova do not). Lane reports that this gives about a tenfold concentration of ova.

Stoll's method of counting ora in the faeces is much used in estimating the heaviness of an infection and in checking the results of treatment. Weigh out accurately 5 Gm. of faeces and add enough N/10 NaOH to bring the volume to 75 cc. Make a uniform suspension by shaking vigorously at least one minute with glass beads. With a suitable pipette remove immediately exactly 0.15 cc., put on a 2 by 4 inch slide and cover with a 22 by 44 mm. cover slip. Using a mechanical stage count all the ova in the preparation. Multiply by 100 to get the number of ova per gram of faeces. This procedure is fairly satisfactory if ova are numerous but fails entirely if they are sparse.

Lanc's D.C.F. (direct centrifugal "floatation") method (1028) is the most efficient. This requires (1) special centrifuge tubes with flat bottom and ground off top; (2) 10 mm. square cover slips 0.5 mm. thick; and (3) special carriers with guards to keep the cover slip from slipping out of position. These are relatively inexpensive and can be used with any centrifuge. Procedure: (1) Measure exactly 1 cc. of faeces (either with Lane's special copper bucket, or by displacement of water in the (graduated) centrifuge tube) and put in the centrifuge tube. (2) Nearly fill the tube with water, stopper, and with the stopper down shake vigorously against the stopper at least 100 times or until a perfectly homogeneous suspension is obtained. (3) Centrifugalize for 1 minute at 1000 r.p.m. (if the bottom of the tube is 6.25 in. from the center of the axis). (4) Decant carefully (about 3% of the ova are lost with this fluid). (5) Nearly fill the tube with 3/4 saturated salt solution (Sp. Gr. 1.150), and resuspend the sediment, avoiding air bubbles. (6) Fill just to the brim with the salt solution. (7) Carefully apply the cover slip which must touch the fluid and fit snugly on the top of the tube without leakage. A small air bubble may be expected. (8) Centrifugalize as before. (0) Lift the coverslip off quickly without tilting (the ova do not adhere to the glass in this salt solution). (10) Mount on a slide in a horizontal position, preferably on two small cones of plasticine so that the fluid does not touch the slide. (11) Count all the ova, using a mechanical stage and a magnification of 150. (12) Add a drop of salt solution so as to refill the tube, as in (7), and repeat the process.

According to Lane, on the average the first coverslip preparation contains 76% of all the ova recoverable, and the second coverslip, 18%. By repeating (7) to (11) twice more, an additional 3% can be recovered, and the final 3% by repeating the entire process with the fluid decanted in (4). Lane reports that this procedure gives more uniform and much higher egg counts than any other method, and that by it infection with a single female worm (if actively laying) can easily be diagnosed.

The same procedure is applicable to *Trichuris* and *Ascaris* ova, but fully saturated salt solution should be used.

Grassi estimated that each gram of faeces contained 15 ova for each worm, of which 25% are males and 75% females. Lane estimated that each female produced about 30 eggs per gram per day. The number of eggs per worm varies greatly, is less in heavy infections, and at best offers only a rough approximation of the degree of the infection.

Cultivation of the larvae (Baermann apparatus as described by Cort et al. 1922) is claimed by some workers to yield positive results more regularly than direct concentration methods. To the tip of a large (8 inch) funnel attach a short piece of rubber

tubing provided with a pinch cock. Fill the funnel nearly to the brim with warm water and suspend on a suitable stand. Cover the funnel with a piece of wire gauze hollowed out so that it dips below the surface of the water. Cover the gauze with a single layer of towel. In the water in the middle of the cloth put the faeces (or soil) to be tested. Incubate for three days or more in a warm room. The larvae work out into the water and sink into the tip of the funnel. If a piece of ice is placed on top of the sample, it will hasten their migration. Draw off a few cc. of water, centrifugalize and examine the sediment for larvae. (Differentiate from Strongyloides larvae.)

The diagnosis can also be made by searching for the adult worms in the faeces after a vermifuge (see Chap. XXXVI). They may be found in cases in which ova are not demonstrable in the faeces by the less efficient concentration methods.

Charcot-Leyden crystals are often present in hookworm stools.

An eosinophilia (10% to 20%, occasionally higher) is usually present but is inconstant and may be absent in the severe cases.

Ancylostoma braziliense (A. ceylanicum) is a parasite of dogs and cats occurring in North and South America, and also in Ceylon, India, Siam and the Philippines. Human infection has been reported from these countries in association with other species of hookworms. The infective larvae may penetrate the human skin and burrow extensively through the subcutaneous tissue, causing a painful itching eruption known as creeping cruption or larva migrans (common in Florida and other southern states as well as in the other regions mentioned). The adult worms (in dogs) are a little smaller than A. duedenale (male 8.5 mm., female 10 mm.) and can be distinguished by the relatively much smaller inner pair of ventral teeth and by the shape of the dorsal ray of the caudal bursa which is deeply cleft and each division again bifurcated.

# Strongylidae

Ternidens deminutus (*Triodonto phorus deminutus*) is a small round worm about the size of a hookworm. The terminal buccal capsule is surrounded by a crown of leaflets, while at the base are three forked teeth guarding the entrance to the oesophagus. The vaginal orifice is near the posterior tip. The ova resemble hookworm ova but are larger, so by  $80\mu$ , more segmented, and have broadly rounded poles and are somewhat flattened on one side. The parasite occurs in several monkeys and has been found not uncommonly in man in South Africa (Sandground, 1931, Blackie, 1932).

Oesophagostomum apiostomum is a round worm about 1 inch long, also resembling a hookworm, occurring in monkeys in the Philippines, China and Africa (Northern Nigeria) where cases of human infection have been found. The worms are found in the caecum and colon, free and encysted in the mucosa. The anterior end presents an ovoid protuberance with a second cuticular inflation just below it, the peristomic collar. The buccal capsule is shallow, with a crown of leaflets at the entrance, and is surrounded by about a dozen pointed plates. The mouth has six papillae.

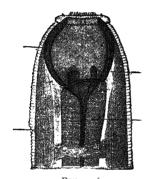
Oes. thomasi, a related species reported in a native of Brazil, has 38 leaflets in the external crown. The large and small intestine showed numerous cysts each containing one worm.

# Trichostrongylidae

Trichostrongylus colubriformis (Strongylus subtilis) is normally a parasite of sheep and goats. It resembles the hookworm in its structure and life cycle. Anteriorly it tapers to a pointed head end which is only one tenth the thickness of the posterior

extremity. The mouth is unarmed. The male (4 mm. long) has two prominent equal spicules. The females (6 mm. long) greatly outnumber the males. The vulva is in the posterior quarter of the body. The ova (42 by 70 to  $90\mu$ ) resemble hookworm ova but are more translucent, and segmentation is much more advanced when passed. The parasite has been found frequently in man (in the small intestine) in Japan, India, Egypt and Central Africa and has been reported in man in Hawaii by Hall. It may be anticipated in the United States, since sheep here are infected. The infection is usually symptomless, although it is reported that heavy infections may cause severe secondary anaemia.

Haemonchus contortus.—This is an intestinal parasite of sheep but has been reported for man. Males are about  $2\frac{1}{3}$  inch long (15 mm.) and females about 1 inch long (25 mm.). The anterior end shows two tooth-like papillae directed dorsad.



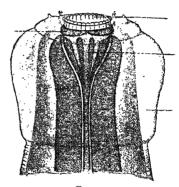


FIG. 116.—Ternidens deminutus. Head showing mouth capsule surrounded by a crown of leaflets. (After Leiper from Mense.)

Fig. 117.—Oesophagostomum. Head showing crown with leaflets at the entrance of the mouth capsule. Note bladder-like thickening of the cuticle around the anterior extremity. (After Raillet and Henry from Mense.)

## Metastrongylidae

Metastrongylus apri (Strongylus apri).—This nematode is a common parasite of hogs, occurring in the bronchi and causing a bronchitis which may be fatal in young animals. It has been reported three times in man.

The male is about 1 inch (25 mm.) long with two long spicules. The female is about 2 inches long and has a sharply hooked posterior extremity with the vulva just beyond the bend. The mouth has two lips each with three lobes. The eggs contain embryos when laid. It probably does not require an intermediate host.

### DIOCTOPHYMOIDEA

Large worms characterized by males having a closed, bell-shaped caudal bursa without rays. Mouth hexagonal with six to eighteen papillae. Oesophagus very long without bulb. Family Dioctophymidae.

## Dioctophymidae

Dioctophyme renale (Eustrongylus gigas), the kidney worm of the dog, may rarely attack man. About 9 authentic cases have been reported. In many of the reported

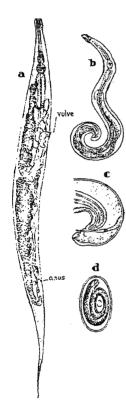


FIG. 118.—Enterobius vermicularis. (a) Female. (b) Male. (c) Coiled lower extremity of the male showing the spicule. (d) Egg. (After Leukart from Brumpt.)

cases fibrinous clots from the ureters or wandering round worms were mistaken for this parasite. It is usually found in the pelvis of the kidney. One or more of the worms may so distend the kidney as to convert it into a mere shell. Pain, haematuria together with the finding of the eggs in the urine make the diagnosis. The eggs are 40 by 65 $\mu$ , brownish yellow, ellipsoidal, with thick shell marked by pitted depressions except at the poles. It is the largest of the parasitic round worms, attaining a length of three feet and the thickness of a man's finger, and has been called the giant strongyle. The male is about 10 inches long.

The collar-like copulatory bursa of the male distinguishes it from *Ascaris*, as does the dark red color. Infection is probably acquired by eating raw fish.

### OXVUROIDEA

Worms with three lipped mouth. Lips simple or indistinct. Oesophagus showing definite posterior bulb. Example, pinworms of family Oxyuridae.

# Oxyuridae

Enterobius vermicularis (Oxyuris vermicularis), the common pinworm or seat worm, is found more frequently in children than in adults. The male is about 4 mm. long, the female a little less than 12 mm. The male has an incurved tail with a single spicule and the female a long tapering tail. The vulva is in the anterior third. These worms have a clear bulbous projection shaped like the mouth-piece of a Turkish pipe surrounding the three-lipped anterior extremity. There is a well-marked bulb-shaped oesophagus.

The eggs have a thin doubly-contoured shell, are plano-convex, 20 by  $50\mu$ , and show a coiled-up embryc. After ingestion of segmented eggs the adults develop in the small intestine, where copulation takes place. The males then die. The fertilized females

then go to the caecum and colon where they remain until the ova have developed. The cycle occupies two weeks. The females then wander to the rectum, and work their way

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out of the anus to deposit their eggs on the skin of the perineum. Marked itching is a common symptom. This is more pronounced at night, and the scratching so induced causes the eggs to be widely spread about the region of the anus. The fingers thus become contaminated with ova which may be carried to the mouth and so cause a fresh infection. The underwear, bedding, towels, wash basins, floors, etc. are frequently contaminated. The possibility that the eggs may be air-borne has been shown. No intermediate host is necessary. About 36 hours outside the body in abundant oxygen is required for completion of development of the infective "tadpole" stage. If reinfection is avoided, the infection dies out spontaneously in a few weeks.

The worms may also wander into the vagina, urethra or under the prepuce.

A knowledge of the life history—the early location in the small intestine and later on in the large—shows that treatment should be dual in its direction—enemata for the gravid female in the rectum and anthelmintics for the young adults in the small intestine. No dependable therapy for pinworm infestation has yet been developed, however.

Ova are rarely found in the faeces, but may be found in scrapings from the skin about the anus, or from under the nails. The diagnosis is preferably made by examining the stools for the white thread-like females which are expelled after a diagnostic dose of calomel and salts. These females which are packed with embryo-containing eggs, may be seen wriggling on the surface of the freshly passed faeces. In handling these worms care must be taken to avoid infection with eggs which may get on the fingers.

In investigations being carried on in the National Institute of Health, under the direction of Dr. E. B. Cram, a survey of various groups in Washington, D. C., shows an incidence of 35 per cent in over 600 persons examined by anal swabs. All positive cases found, but only part of the negative cases, were followed by examination of the families of which the individuals were members, since pinworm infestation is usually a familial affair, with commonly about three fourths of the members of a family infested. The high incidence in Washington indicates that when pinworms are given attention in the United States, it will be found that the situation here is in line with that stated by the best informed parasitologists, i.e., that pinworms are the most common and widely distributed of human helminth parasites.

The eggs of  $Heterodera\ radicicola$ , a harmless nematode which is parasitic on certain food plants (tubers), occur in the faeces if such infected plants are eaten. They were once reported as a supposed new parasite, "Oxyuris incognita." They are large, 40 by  $90\mu$ , asymmetrical, and contain a morula with a large, highly refractile, greenish body at each pole.

#### ASCAROIDEA

These have a mouth commonly provided with three prominent lips supplied with papillae, one dorsal and two ventral. Oesophagus muscular and usually without posterior bulb. As a rule an intermediate host is not required. Example, common large intestinal ascarid of family Ascaridae.

#### Ascaridae

Ascaris lumbricoides, the common round or eel worm, is probably the most common parasite of man, especially of children. It is also found

in the gorilla. It occurs throughout the world, in temperate as well as tropical regions, and has been reported from the arctics.

Morphology.—The worms are cylindrical, tapering to a blunt point at each end, and are yellowish grey to light brown in color. The body is transversely striated. The females are usually 20 to 36 cm. long and 4 to 5 mm. in diameter; the males are more slender, and from 15 to 30 cm. long. The mouth is provided with three papillae-like lips with finely denticulate margins, marked off from the rest of the body by a sharp constriction. The cloaca is near the posterior tip, and from it in the male projects two large lance-like copulatory spicules. The posterior extremity of the male, unlike the female, is curved ventrally, and has seven pairs of post-anal papillae. The uterus

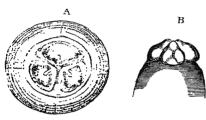


Fig. 119.—Anterior extremity of Ascaris lymbricoides; A, seen from front; B, seen from dorsal surface. (Tyson after Railliet.)

consists of long whitish convoluted threadlike tubes terminating in oviducts which lead to the vulval orifice on the ventral surface at the junction of the anterior and middle thirds of the body.

After fecundation the females discharge enormous numbers of ova, estimated at 200,000 each daily. These are elliptical, 40 to  $50\mu$  by 60 to  $75\mu$ . They are provided with a thick, smooth, translucent inner shell and a rough, mammillated outer coat which is sometimes lost. The contents are granular, not segmented, and usually show a clear crescentic area at

each pole. Eggs which are deposited unfertilized are markedly abnormal in their appearance, often longer, irregular in shape and sometimes grotesquely misshapen, with structurcless granular contents, so that they may be mistaken for vegetable cells.

The adult worms live in the upper small intestine. As a rule the number present is small, but rarely many hundreds may be present. If numerous they tend to aggregate in clumps which may be demonstrable in roentgenograms as filling defects, and may cause intestinal obstruction. Occasionally worms may migrate up or down the intestinal tract, being passed per anum or vomited when they reach the pharvnx. They may penetrate into any accessible passage or space and cause bizarre and sometimes serious local disturbances; e.g., into the appendix, bile ducts, gall bladder, pancreatic duct, nose, sinuses, middle ear, larynx. Guiart considers it probable that Ascaris may suck blood and cause intestinal ulceration and bacterial infection. They may perforate the intestine and cause peritonitis, especially through an ulcer or a sutured surgical incision; a real danger in countries like Guam or the Philippines in which the parasites abound. They may cause general systemic disturbances, such as pruritus, anaemia, vertigo or convulsions, which have been attributed to a toxin ("ascaron"). In some cases outspoken allergic phenomena appear: conjunctivitis, urticaria, asthma, even oedema of the glottis after vomiting a worm. Such cases give a positive cutaneous reaction to extracts of the worm.

Life history.—There is no intermediate host. The ova in fresh faeces are unsegmented and non-infective. In the presence of warmth (but not at body temperature), a little moisture and oxygen, segmentation occurs outside the body, and development

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of the embryo is completed after an interval of from 10 to 40 days or more, (rarely less than 30) depending upon the temperature (optimum 30°C.). Normally the larva remains within the shell until ingested. However, hatching can be induced by first desiccating and then moistening the ova, and the larvae may live for several weeks in moist earth (Kondo). The ova are very resistant to the usual chemical disinfectants, to cold (down to  $-r_5$ °C.) and to desiccation at moderate temperatures, but are killed by desiccation in hot weather. Eggs have remained viable four years when stored moist at ice box temperatures, and two years when dried.

It is generally believed that infection normally occurs by swallowing "embryonated" ova. Infection is heavy in regions in which soil pollution is common, or in which human faeces are used as fertilizer. Ova may be conveyed to the mouth by dirty fingers (especially in children) or by contaminated water, green vegetables or other food, and possibly by inhalation. The larvae (0.25 mm. long) are liberated in the contents of the small intestine. They penetrate the wall of the gut (Stewart, 1916) and pass to the liver, usually through the portal blood stream, a few perhaps by direct migration through the peritoneal cavity. After three to four days they reach the lungs. Here they penetrate the walls of the capillaries and alveoli, and after a further period of development of eight days they enter the air passages, pass by way of the trachea to the pharynx and are swallowed, as in the case of the hook worms. While in the lung, if the infection is heavy, the parasites give rise to a bronchitis with considerable bleeding, or even to an "ascaris pneumonia" accompanied by fever and eosinophilia. They may be demonstrated in the sputum. This passage through the lungs seems to be essential for the development of the worm.

On reaching the intestine the larvae (2.0 to 3.0 mm. long) develop into adult forms after a period of 8 to 10 weeks. The life span of the adults has been estimated at about a year.

This cycle has been demonstrated by feeding mature A. lumbricoides ova to rats, guinea pigs and hogs. In these animals the ova hatch, and the larvae migrate to the liver, lungs and back to the intestine (although in such unsuitable hosts development usually does not go on to the production of mature adults). The same appears to be true in Koino, e.g., swallowed 2000 mature Ascaris ova and developed pronounced symptoms of lung involvement with bloody sputum containing larvae. Fifty days later, following an anthelmintic, 667 worms were recovered. Yoshida swallowed larvae taken from the lung of a guinea pig and ten weeks later showed ova in the faeces. In such feeding experiments, however, the proportion of larvae which succeed in establishing themselves in the intestine is usually very small, and occasionally none have done so. This fact and other indirect epidemiological evidence led Lane to suggest that infection may occur by inhalation of desiccated ova in dust. The ova may hatch on the moist mucous membranes of the upper air passages and the larvae penetrate directly into the blood stream without being swallowed. There is some evidence that under experimental conditions hatched larvae may penetrate the skin, but this is not known to occur under natural conditions.

The round worm of the pig, A. suilla, is morphologically identical with A. lumbricoides, but appears to be biologically distinct. Feeding experiments indicate that neither parasite will develop to maturity as a rule except in the homologous host. Lane (Critical review, 1934), however, doubts the validity of this evidence, and believes the question is still open. Ransom showed that a serious lung disease (thumps) of little pigs is caused by Ascaris larvae passing through the lungs.

Laboratory diagnosis depends upon finding the ova in the faeces, or the adults after a vermifuge. It is usually easy since the typical ova are unmistakable and numerous (about 2000 ova per gram for each female). Ova which have lost the outer shell, or unfertilized ova may present difficulties.

Toxascaris leonina (Belascaris cali) and Toxocara canis (Toxascaris canis), the common ascarids of the cat and dog respectively, have been reported for man. They are much smaller than A. lumbricoides averaging about three inches in length and are characterized by wing-like projections from the anterior end by reason of which they are called the arrow-headed ascarids. The eggs are thick shelled and somewhat similar to those of A. lumbricoides.

### SPIRUROIDEA

Members of this superfamily may be plump, resembling Ascaris, or long and filiform, resembling Filaria. Lips when present, two, paired, simple or trilobate. Oesophagus practically always divided tandem. Vulva more equatorial. Family of interest: Spiruridae.

# Spiruridae

Physaloptera caucasica (P. mordens) is normally a parasite of monkeys and has been reported in man in eastern and northern Africa where it is said to be fairly common. The mouth is provided with two large, equal, laterally placed lips, each having two papillae and armed with teeth. The male (15 to 50 mm. by about 0.8 mm.) has a lance-shaped posterior extremity with two unequal spicules. The female (25 to 100 by about 2 mm.) has a pointed tail, and a vulva opening in the anterior part of the body. The

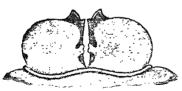


FIG. 120.—Physaloptera mordens. Mouth parts showing the two lateral lips, each with papillae and teeth. (After Leiper from Mense.)

ova  $(35 \text{ by } 45\mu)$  have a thick smooth shell. The worms are found in the stomach and small intestine. The life history is not known.

Gongylonema hominis (probably G. pul-chrum) is a thread-like worm much like a filaria, 0.5 mm. in diameter and from 3 to 15 cm. long. The cuticle of the oesophageal zone is provided with eight rows of shield-like elevations, quite distinct from the bosses of Loa. The males have caudal alae and unequal spicules. Vulva is somewhat preanal. Infection is acquired by swallowing cockroaches, croton

bugs, or various beetles, the intermediate hosts. They normally occur in rodents, cattle, sheep and goats. At least seven cases of human infection have been recorded (Italy, southern United States), the worms living in the buccal mucous membranes in which they migrate actively. A related species (*G. neoplasticum*) sometimes causes carcinoma in the stomach of the rat.

### Gnathostomatidae

A number of cases of human gnathostomiasis mainly due to *Gnathostoma spinigerum* have been reported, chiefly from Siam. This species is normally parasitic in the stomach wall of wild and domestic cats, forming hard cystic tumors one inch in diameter

and causing grave disturbances. The worm is about 2.5 cm. long and 2 mm. in diameter. The cephalic end is expanded into a globular swelling armed with eight rows of thorn-like hooks. The eggs (in faeces) have a plug at one end like a bottle stopper and are not segmented when deposited. They develop into larvae in a cyclops. The parasite is not adapted to man as a host, and in most cases immature worms which presumably have "lost their way" have been found in the subcutaneous tissues in which they may cause abscesses. Chandler found that encysted gnathostome larvae which are infective for cats are common in snakes in south-eastern Asia, and they probably occur in other animals. It is believed that human infection might be caused by eating raw snakes, a practice indulged in by some wild tribes in this region.

## FILARIOTDEA

This superfamily is of the greatest importance to man. Much confusion still exists as to the adult stage of these parasites. Hence anyone finding adult filariae should preserve them for careful study, fixing them in hot 5% glycerin alcohol (alcohol 70%), and subsequently mounting in glycerin gelatin. Formalin should not be used except for a very brief period (two to six hours), and then the worms should be transferred to lacto-phenol.

### Filariidae

These are thread-like worms which are found usually in the lymph vessels and glands, the connective tissue, and about the body cavities.

They have a lipped or simple mouth and a filariform oesophagus. The male has an incurved tail with preanal and postanal papillae, which may be even corkscrew-like as in *Dirofilaria immitis*. The spicules are unequal, or there may be but one. The females are viviparous. The vulva is at or near the anterior end, and the uterus is double.

Wuchereria bancrofti ( $Filaria\ bancrofti$ ) is the most important of the filarial worms. Infection with it is common in south China, India, the West Indies, and the Pacific Islands, especially Samoa, where at least 60% of the inhabitants are infected. It is widespread throughout tropical and subtropical regions from southern Spain and South Carolina to Australia.

The adult worms live in the lymphatics of the trunk and extremities, particularly the lower abdomen and inguinal regions. At times the fine white worms may be seen as writhing coils within the lymphatic glands. The sexes are usually found together, often coiled up in masses in cyst-like dilatations of the lymphatic vessels. The females are about 75 mm. long and 0.25 mm. broad, the males about 40 mm. by 0.1 mm. The head is bougie-shaped, with two rows of papillae. The tails of both sexes are incurved, but that of the male is more so and has 15 pairs of minute postanal papillae and two unequal spicules. The vulva opens 1.2 mm. from the anterior end. There are two uterine tubules.

The females give birth to enormous numbers of larvae which make their way through the lymphatics to the blood stream where they may be readily found. They were first described in the blood by Lewis under the term *Filaria sanguinis hominis*. Filarial larvae are often spoken of as Microfilaria, followed by the specific name of the parasite in question (bancrofti, etc.). The larvae of this species are about 300 by 7.5 $\mu$  in size.

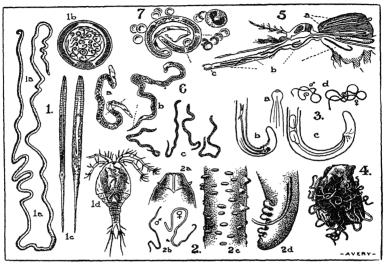


FIG. 121.—(1a) Adult female Guinea worm (Dracunculus medinensis) showing anchoring hook at posterior extremity. (1b) Cross section of female Dracunculus showing uterus filled with embryos. (1c) Striated embryos of the Guinea worm. (1d) Cyclops coronatus, the minute crustacean which serves as the intermediate host of D. medinensis. (2a-2d) Anterior and posterior extremities of Loa loa. (2c) Section showing tuberculated cuticle. (2b) Male and female L. loa, natural size. (3a) Bulbous anterior extremity, Wuchereria bancrofti. (3b) Tail of male. (3c) Tail of female. (3d) Male and female, natural size, of W. bancrofti. 4. Tumor mass of 0 rolevulus laid open. 5. Mosquito showing filarial embryos in thoracic muscles (a) and in lahium (b). The labella which are separated from the labium by Dutton's membrane are seen at (c). 6. (a) Embryo of W. bancrofti (b) embryo of L. loa showing filling of tail end with cells. 7. Microfilaria of W. bancrofti in blood. Dotted lines show location in break in cell column and V. spot. (Not drawn to scale.)

They are actively motile in fresh preparations and easily found by the commotion they cause among the adjacent red cells by their lashing movements. They are enclosed in a thin translucent sheath which projects somewhat beyond the ends of the body of the larva. Some observers have described (but others deny) a very slender lancet at the cephalic end, which is alternately extended and retracted so as to jostle the adjacent red cells. In preparations stained by dilute Giemsa stain columns of minute nuclei can be made out in the body of the larva. These are interrupted (1) by the neural ring (a break in the cell columns)  $50\mu$  from the head end; (2) by the V-spot, marking

# PERIODICITY OF FILARIAL LARVAE

the site of the excretory cell and pore,  $90\mu$  from the head; and (3) in the posterior end by a solid body (the "internal body") and just behind this by the large genital cell, three small genital cells and the anal cell. The nuclei do not extend quite to the tip of the tail. The tail is pointed and the organism gracefully curved. For differences from those of other species, see Key.

In most regions the larvae show a remarkable nocturnal periodicity in their appearance in the blood stream. During the day they are sparse or absent. Towards evening their number begins to rise, reaching a maximum about midnight or 2 A.M. when there may be 300 to 600 in one drop of blood, and dropping off rapidly towards morning. If the host sleeps during the day and works at night, there is a gradual reversal of the periodicity. The mechanism of this phenomenon is in controversy. Some believe that it is due to a migration of the larvae back and forth from the internal to the peripheral vessels. Manson reported that in the case of a patient who committed suicide



Fig. 122. - Larva of Loa loa above; A. perstans below. (From Greene, after Fülleborn.)

during the day, the larvae were largely in the small vessels of the lungs and kidneys and in the large arteries, to the walls of which they adhered in enormous numbers. Lane has since suggested that it is due to a (simultaneous) periodic cyclical parturition by the female worms, and that the larvae are removed from the blood and destroyed the following day. The observations of O'Connor on worms removed at operation indicate that such a cyclical parturition does take place (about midday).

Whatever the mechanism may be, it appears to be an adaptation to the feeding habits of the most common intermediate host, Culex quinquefascialus (C. fatigans), which feeds at night. In Fiji and many Pacific Islands there is no such periodicity, although the parasites seem morphologically identical with the usual W. bancrofti. O'Connor showed that the distribution of the non-periodic strain of W. bancrofti corresponds exactly with that of Aedes variegalus (Stegomyia pseudoscutellaris), the intermediate host in this region, which bites by day. It has been suggested that a poison introduced

by the biting mosquito supplies the stimulus for the migration of the larvae (or the cyclical parturition). While direct evidence is lacking, Strong reported for another



Fig. 123.—Male (a) and female (b) of Wuchereria bancrofti. Natural size. From Greene, after Manson.)

parasite related to this one that the bite of a simulium fly exerts a positive chemotactic effect on *Onchocerca* larvae in the lymph spaces of the skin.

The parasites are long lived; the number of larvae in the blood and the regular nocturnal appearance of the larvae in the blood may be maintained for as long as 12 years. The length of life of the individual larvae is not definitely known. There is evidence that they can live much more than

24 hours, although in some cases in which the adult worms have probably died as the result of an attack of lymphangitis, the larvae have disappeared quite abruptly from the blood.



FIG. 124.—Section of Aedes variegatus, showing filariae in thorax on tenth day of development, travelling forwards into proboscis. (By permission from Manson's "Tropical Diseases.")

Intermediate host.—In addition to the two species mentioned many other species of mosquitoes serve for the complete or partial development of the larvae, including Culex pipiens (China) and several species of Anopheles.

Life History.—Upon reaching the stomach of the mosquito the sheath of the larva becomes fixed in the viscid blood contents, and the larva by its active movements

gradually frees itself from the sheath. The larvae then bore their way through the wall of the stomach into the thoracic muscles, which they reach within 24 hours. They there undergo growth and development for a period of about 3 weeks. They then work their way forward into the fleshy labium of the proboscis. At this stage they measure about 1.5 mm. by  $20\mu$ . When the mosquito bites they work their way out onto the skin by piercing Dutton's membrane, a thin partition separating the labium from the two terminal processes, the labella. They are not directly inoculated by the bite, but pierce the intact skin, enter the circulation and are carried to the lymphatics. Here they develop into adult worms, a process which probably requires many months. The mosquito is injured by the parasites and may die if heavily infected.

Clinical Symptoms.—In a majority of the cases the infection is symptomless, even though large numbers of larvae are present in the blood. In some cases serious disturbances arise as a result of obstruction of lymphatic channels. This may be due to the mechanical presence of the worm, alive or dead, or extruded ova; or to chronic inflammation and fibrosis excited either by the worms or their products or secondary bacterial infection. Attacks of acute (bacterial) lymphangitis are common and are often followed by the death of the parasites which gradually become calcified, and may then be demonstrable in roentgenograms.

The obstruction commonly results either in *lymphatic varices*, most often in the scrotum, inguinal region or lower abdomen; or in *elephantiasis*. Rupture of a varix into the urinary passages gives rise to *chyluria*; into the peritoneum, to *chylous ascites*. In the case of lymph varices the larvae can usually be demonstrated in the accumulations of lymph as well as in the blood stream, but in most cases of elephantiasis they are not found in the blood. This may be due to the death of the adult worms or to complete obstruction of the lymphatics which prevents the larvae reaching the blood stream.

Laboratory Diagnosis.—Diagnosis usually depends upon demonstrating the larvae in the blood. While it may be possible to find them in fresh preparations, as a rule thick films should be made and stained (Giemsa) as for malaria. The larvae in a non-motile state may be concentrated by laking blood in five volumes of 2% acetic acid and examining the sediment after centrifugalization. Larvae may be sparse and negative results are not conclusive. Blood should be obtained at midnight and also during the day.

In hydrocele fluid or fluid aspirated from a varix, and in chylous urine or ascitic fluid larvae are often present and may be demonstrated in the sediment or clot. If a bit of absorbent cotton is dropped into such fluid and allowed to settle to the bottom of the container, the larvae will be entangled in the meshes and easily demonstrable.

Complement fixation.—Fairley showed that a positive reaction could be obtained in most cases of infection with this and other related filariae (a group reaction) by using an antigen made from *Dirofilaria immitis*, the heart worm of the dog. The worms are thoroughly desiccated and ground, and 0.5 Gm. of the powder is extracted with 50 cc. of 98% alcohol for

24 hours at 37°C., shaking occasionally. This is concentrated by putting it in a water bath at 40°C. and bubbling air through it until it becomes turbid. The turbidity is removed by adding alcohol to a volume of 25 cc. It is preserved on ice in the dark. The usual technique as described under the Wassermann reaction is followed.

A cutaneous test has also been devised, using a 1% saline extract of this powder (2 hours at 37°C.) which has been cleared and sterilized by suc-



FIG. 125.—Loa loa in the subcutaneous tissue, twice normal size. (From Greene, after Fülleborn.)

cessive filtration through paper, a Buchner funnel and a Seitz filter. Fairley injected 0.25 cc. of a 0.1% extract and regarded the prompt or delayed appearance of a wheal over 2 cm. in diameter as positive.

Loa loa (Filaria oculi, F. diurna) occurs commonly in western and central Africa, especially in the Congo region, and also in the West Indies. The male is about 30 mm. by 0.4 mm., the female longer (up to 70 mm.) and 0.5 mm. broad. The cuticle is studded with numerous rounded bosses about  $15\mu$  in diameter and  $10\mu$  in elevation. The anterior extremity is shaped like a truncated cone. The males have four pairs of papillae increasing in size from the postanal to the preanal, which are large and pedunculated. There are two short unequal spicules. The females are viviparous. The vulva opens 2.5 mm. from the anterior end.

The larvae closely resemble those of the W. bancrofti, but in stained preparations can usually

be distinguished by these points: Loa loa shows angular curves and an acute bend at the tail. The nuclei are coarser, extend to the tip of the tail, and end squarely at the head instead of in a curve. There is no "internal body." They have a diurnal periodicity even more clear cut than the nocturnal periodicity of W. bancrofti. The intermediate hosts are the mangrove flies, Chrysops dimidiala and C. silacea, in which a cycle of development occurs similar to that of IV. bancrofti in the mosquito.

Development in the infected human being is very slow. From three to six years may elapse before larvae appear in the blood. There are no systemic symptoms. The adult worms live in the connective tissue in which they wander extensively and may appear in the superficial tissues, occasionally about the eye lid or under the conjunctiva, where they cause more or less serious local irritation. They may cause areas of localized oedema, particularly on the hands and arms. These Calabar swellings are about the size of a hen's egg, appear abruptly, are painless, do not pit on pressure, and subside after about three days. Some regard them as allergic phenomena. Larvae may not be demonstrable in the blood in such cases, even in those from which worms have been removed.

Acanthocheilonema perstans (Filaria perstans, Dipetalonema perstans) occurs in tropical Africa and in South America. The adults are found chiefly in the mesentery

and retroperitoneal connective tissue. They resemble those of the preceding species but have a smooth body and an incurved tail, the extremity of which has two triangular appendages giving it a bifid appearance. The larvae are smaller than the preceding (about  $200\mu$  by  $4.5\mu$ ). They have no sheath. The tail is blunt. They show no periodicity. The intermediate host is a midge, *Culicoides austeni* and *C. grahami*. The infection appears to be symptomless.

Onchocerca volvulus (Filaria volvulus) is common in scattered areas in central Africa, especially the Belgian Congo, and in the mountainous regions of western Guatemala and southern Mexico, where it was described as O. caecutiens Brumpt, the "blinding filaria." The adult worms are found in the subcutaneous lymphatics, usually enclosed in fibrocystic nodules from 2 mm. to 30 mm. in diameter. Several worms, male and female, may usually be found in each nodule, in which they are coiled up and imprisoned in a mesh of connective tissue.

The female parasite is up to 35 cm. long and 0.4 mm. in diameter, the males 3 to 4 cm. by 0.15 mm. The cuticle is striated. They are viviparous. The larvae are very numerous in the cystic fluid and in the lymphatics of the skin, but they rarely enter the blood. They are about the same in size as W. bancrofti but have no sheath. The tail is pointed.

The intermediate host in Africa is the jinja fly, Simulium damnosum, and probably S. norvei; in America, Eusimulium avidum, E. ochraceum, and E. mooseri. The occurrence of related species of Eusimulium in the southwestern United States suggests the possibility that the disease might become established if it were introduced into this region.

Clinical Symptoms.—Several months are required for the development of nodules after an infecting bite. Their formation is often associated with recurring periods of considerable pain and fever which have a curious tendency to come at intervals of two weeks. In Africa the nodules are usually over the ribs, in the axillae, elbows and popliteal spaces; in America, about the head. The larvae cause irritation of the skin varying in degree, often intense itching, induration and lichenoid eruptions. In the Congo, cases of elephantiasis have been attributed to this species, the larvae having been found in the lymph aspirated from the swollen tissues. The larvae appear able to spread in the skin for a considerable distance from the nodules, and in chronic cases (at least 5 years' duration) may penetrate into the tissues of the eye, causing intense irritation, lachrymation, photophobia, a punctate keratitis with iritis and uveitis, a choroiditis and possibly a retrobulbar neuritis. Larvae have been reported in the optic nerve itself. Blindness eventually occurs. The earlier observations indicated that the ocular manifestations subsided promptly after systematic excision of all the nodules, thus cutting off the supply of microfilariae. Subsequent observations, unfortunately, have not confirmed this. First reported by Robles (1916), this condition has been described at length in the monograph of Strong et al (1934).

Although involvement of the eye has been considered peculiar to the American form of the disease; Hisette (1932) has described identical ocular lesions in the Congo, and reported finding them present in more than half the population in certain stricken native

villages. Bryant (1935) has reported onchocerciasis in the Sudan and believes it to be the major cause of "Sudan blindness." Although some of the cases showed the typical punctate keratitis, most of them showed an optic atrophy (retrobulbar neuritis) with no larvae in the ocular tissues. However, in certain districts in which blindness was common, 58% of the victims showed nodules or other evidence of Onchocerca infection, whereas these were present in only 9% of the general population.

Diagnosis may be made by demonstrating the larvae and adult worms in an excised nodule (these can be enucleated easily). The larvae are very numerous in fluid aspirated from a nodule. A piece of skin (preferably the superficial layers only) may be

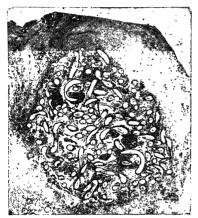


FIG. 126.—Section through a nodule of Onchocerca volvulus. from Mayer.)

excised, teased in salt solution on a slide and examined for motile larvae, or films may be stained by Giemsa's method. If the eye is involved larvae may be demonstrated similarly by excising a bit of bulbar conjunctiva or by examining fluid aspirated from the anterior chamber. They may be seen moving in the cornea or aqueous fluid by using an ocular microscope.

A positive complement fixation test may often be obtained by using Fairley's antigen, or preferably (Van Hoof) an antigen made from worms dissected out of *O. volvulus* nodules. Intracutaneous tests are said to be less satisfactory.

Mansonella ozzardi (Filaria ozzardi, F. demarquayi) occurs in the West Indies and parts of South America. The adults live in the mesentery and visceral fat and appear to cause no symptoms. The unsheathed larvae resemble M. perstans but are somewhat smaller and have a pointed tail. They show

no periodicity. The intermediate host is a midge, Culicoides furens.

Filaria malayi (Brug, 1927), of which only the larva is known, occurs in the Malay Archipelago and in Ceylon. The larvae are  $250\mu$  by  $5\mu$ , and possess a sheath projecting much beyond the caudal tip. The tail is acutely pointed and shows a deeply staining nucleus in the tip. Nocturnal periodicity has been reported in Ceylon. Several species of *Mansonoides* mosquitoes serve as intermediate hosts.

Agamofilaria streptocerca is an unsheathed larva found in the skin of natives in the Gold Coast and Cameroons. It resembles *Onchocerca*, but the tail is curved like the handle of a walking stick. Neither the adult form nor the vector is known. It appears to cause no symptoms.

The points useful in the differentiation of filarial larvae are:

- 1. Presence or absence of periodicity of larvae in peripheral circulation.
- 2. Presence or absence of a sac sheath around the larva.
- 3. Accurate measurements.
- 4. Shape and description of head and tail ends.
- 5. Character of movement.
- 6. Location of V spot and break in cell column in stained specimens.

Loa loa.

## KEY TO FILARIAL LARVAR

- I. Present in peripheral blood.
  - A. Sheath present.
    - 1. Nocturnal periodicity (usually).

      - b. Tail pointed, straight; an elongated nucleus in tip. Sheath much prolonged beyond the tail. No nuclei at anterior end. Size 250 by 5 to 6μ.
         F. malavi.
    - 2. Diurnal periodicity.
      - a. Tail pointed, sharply flexed; nuclei coarse, extending to tip. Sheath moderately prolonged beyond tail. Break in cells 40μ and V-spot 60 to 70μ from head. Irregular sharp flexures. Size 250 to 300 by 7.0μ.
  - B. Sheath absent. No periodicity.
    - 1. Tail sharp, straight; nuclei not reaching tip. Size  $200\mu$  by  $5\mu$ ...M. ozzardi.
    - Tail blunt, straight; nuclei reaching tip. Break in cells 34μ and V-spot 50μ from head. Lashing progressive movements. Size 200μ by 4.5μ (variable).
- II. Present in lymph spaces, not in blood. (No sheath, no periodicity).

# Dracunculidae

These differ from the Filariidae in that the vulva appears to be atrophied in the gravid female, the anus is absent, and the females are enormously larger than the males.

**Dracunculus medinensis** (Filaria medinensis), the Guinea worm or Medina worm, is common in parts of India, Africa and Arabia and has become indigenous in parts of the West Indies, Brazil and Guiana. A parasite morphologically identical with D. medinensis has been found in various carnivores (fox, raccoon, mink) in the United States and appears to be established in this country, but no human cases have been reported. Dogs may be infected, naturally and artificially.

The female is thread-like, cylindrical, 1.6 mm. in diameter, and may reach a length of one meter. It lives in the subcutaneous tissue and intermuscular connective tissue, particularly of the lower extremities. The tip of the tail is bent, forming a sort of anchoring hook. The mouth is terminal. The uterus is a continuous tube occupying the greater part of the body and filled with sharp-tailed, transversely striated, unsheathed embryos 600 by 20 $\mu$ . These are discharged as the result of a gradual pro-

lapse of the uterus through the ruptured head. After discharge, the worm dies. The males, obtained by Leiper from monkeys experimentally fed with infected Cyclops, were less than an inch in length.

The worm causes no *symptoms* during its development, but when mature and fecundated it burrows down into the region of the ankle joint or elsewhere, the head pene-

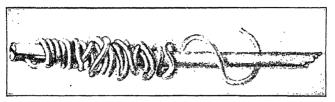


Fig. 127.—Guinea worm. Rolled on a stick for gradual extraction. (From Greene's "Medical Diagnosis.")

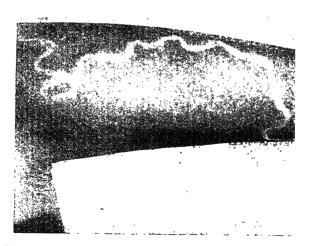


Fig. 128.—Female guinea worm lying under the skin of the forearm. (By permission from Manson's "Tropical Diseases.")

trates into the dermis, and over it appears a cutaneous blister which ruptures leaving a painful ulcer. This is accompanied by burning and itching and often by systemic symptoms, nausea, vomiting, diarrhoea, urticaria, asthma and fainting, which suggest an anaphylactic reaction. The head of the worm may project slightly from this opening. In attempting to remove the worm great care must be taken to avoid tearing or crushing it. If the body contents escape into the tissues they excite a very violent and serious inflammatory reaction.

# GENERAL FILARIAL TABLE

Adults		Larvae	Remarks
Wuchereria bancrofti (Filaria bancrofti).	Male 40 by 0.1 mm. Female 90 by 0.28 mm. Smooth cuticle. Bulbous anterior extremity. Occupy lymphatic glands and vessels.	Graceful curves; tail rather straight. Sheathed, 300 by 7.5 microns. Distance from head to V spot 90 microns; to break in cells 50 microns. Cells in head end form a curved line. Terminal cells do not fill up tail end. Nocturnal periodicity in peripheral circulation.	Transmitted by mosquitoes: Culex: Aedes, Anopheles. Causes elephantiasis, lymph scrotum, chyluria, etc.
Loa loa.	Male 27 by 0.3 mm. Female 55 by 0.4 mm. Cuticle tuberculated. Anterior extremity like truncated cone. Wanders in subcutaneous tissues.	Angular curves; acute bend at tail. Sheathed, 240 × 7 microns. Distance from head to V spot 65 microns; to break in cells 40 microns. Tail is completely filled up with terminal cells. Diurnal periodicity in peripheral circulation.	Transmitted by species of a biting fly —Chrysops. Causes Calabar swellings. Worms often visit ocular region.
Acanthocheilonema perstans.	Male 40 by 0.07 mm. Pe- male 75 by 0.1 mm. Cuti- cle smooth. Anterior ex- tremity club shaped. Tip of tail shows two trian- gular processes. Found about root of mesentery.	Without sheaths, 200 by s microns. Posterior two-thirds tapers to blunt ending. Cells to end of tail. Distance from head to V spot 49 microns; to break in cells 34 microns. Persists in circulation both day and night.	Transmitted by midges: Culicoides. No pathogenicity.
Mansonella ozzardi (Filaria ozzardi).	Female only is known; 65- 80 mm. in length; a pair of fleshy papillae at tail end. Lives in retroperi- toneal tissue.	Without sheaths, $200 \times 5\mu$ . Tail sharp pointed; cells not to end of tail.	Transmitted by midges: Culicoides furens. No pathogenicity.
Onchocerca volvulus. (O. caecu- tiens).	Males 30 by 0.15 mm. Females usually fragmented, up to 35 cm. by 0.4 m. Cuticle striated. Found coiled up in cyst-like tumors under the skin.	Without sheaths. 250 by 7.5µ. Found in cyst-like spaces in nodules and in cutaneous lymph spaces. very rarely in blood.	Transmitted by flies: Simulium and Eusimulium. Causes small subcutaneous cystic tumors, fever, skin eruptions, punctate keratitis, blindness.
Dracunculus medinensis.	Male from Leiper's monkey 22 mm. Female 80 to 90 cm. long by 1.6 mm. wide. Smooth white body. An- choring hook at tail end. Female lives in subcuta- neous tissue of lower ex- tremity.	Without sheaths. 600 × 20 microns. Tapering outline; gut present. Long slender tail. Cuticle striated. Extruded from break in skin of patient. Not present in blood or tissues.	Larvae develop in Cydops. Man infected by drinking water containing Cyclops. Causes "Dracontiasis."

When the ankle is immersed in water, the worm discharges a drop or two of milky fluid containing multitudes of coiled larvae with the slender sharply pointed tails straightened and projecting in a characteristic manner. In water the larvae quickly begin to swim about and are swallowed by Cyclops quadricornis or related species, in which they undergo development during a period of four to six weeks, reaching a length of about 1 mm. They then remain latent in the thoracic muscles until the Cyclops is swallowed in contaminated drinking water. Leiper showed that HCl in the concentration present in gastric juice kills the Cyclops but makes the Dracunculus larvae very active. They doubtless bore through the stomach wall and make their way to the connective tissue. Development to maturity in man is believed to take about a year.

As a prophylactic measure Leiper suggested pumping steam into the water of wells containing Cyclops to destroy them.

### ACANTHOCEPHALA

These are called thorn-headed worms because they possess a proboscis which projects anteriorly like a little peg and is armed with several rows of hooks which are directed backward and enable the parasite to attach itself to the intestinal wall. The worms absorb nourishment through the general body wall, there being no alimentary canal or mouth.

Macracanthorhynchus hirudinaceus (Gigantorhynchus gigas) is normally an intestinal parasite of hogs. The male is 2 to 4 inches long, the female 10 to 18 inches. The body shows transverse rings and resembles Ascaris but is more white in color. The eggs, which are brown in color and about  $100\mu$  long, contain hooked embryos. The intermediate hosts are larvae of June bugs and related beetles. Human infection is said to be common in south Russia. Moniliformis moniliformis (Gigantorhynchus moniliformis), an intestinal parasite of rats, has been reported in man in a few cases. It might be contracted by eating death watch beetles, as is sometimes done to improve the complexion. The male is 2 inches long, the female 4 to 10 inches. The proboscis has 12 to 15 rows of hooks. A beetle, Blaps mucronala, and the cockroach, Periplaneta americana, are the intermediate hosts.

### Annelida

Leeches belong to the higher worms, class Hirudinea. Members of this class are parasitic and do not possess chaetae, but move about by means of a sucker at the posterior end.

They have a rather oval body marked by numerous rings, and have a well developed muscular system which enables them actively to contract and extend. There is a sucker at the anterior end; within this is the mouth leading to the pharynx which by the action of its muscular walls serves as a pumping organ. The salivary glands, situated inside the mouth cavity, secrete the fluid which prevents coagulation of the blood. The mouth may or may not be provided with cutting jaws. In *Hirudo* there are three semicircular jaws, the arched surfaces of which are beset with from 50 to 100 sharp teeth. The mark of a leech bite is triangular. When a leech has gorged itself, it becomes detached from the skin of its victim, but the effect of the salivary secretion in retarding

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coagulation is of some duration so that the wound continues to bleed. With some of the leeches the wounds frequently become infected and ulcers, which may prove serious, often result. This is particularly true with the *Haemadipsa zeylanica*. This species is a land leech but requires abundant moisture. Most leeches live in water.

As a rule leeches are hermaphroditic and reproduce by depositing so-called cocoons, which are rounded bodies surrounded by a shell and containing eggs in an albuminous matrix.

Hirudo medicinalis.—This is the leech used medically for the abstraction of blood. They have a secretion which prevents coagulation of the blood so that when they are removed the wound still continues to bleed. These leeches are about 4 inches long and of a grayish-green color with dingy red longitudinal stripes on the dorsal surface and with a dark-green ventral surface.

Limnatis nilotica is found in many parts of northern Africa, Palestine and adjacent regions. The young leeches which are only about 3 mm. long gain access to the mouth when contaminated water is drunk. They attach themselves to the mucous membrane of the mouth, nose, larynx or even trachea, remaining there several weeks until they reach adult size (up to 10 cm. long and 1.2 cm. wide). They may cause headache and obstinate bleeding, often resulting in severe and even fatal anaemia. The dorsal surface is greenish brown with orange-brown borders.

Haemadipsa zeylanica.—These are land leeches found in India, Philippines, Australia, and South America. They are only about r inch (25 mm.) long and are slender. They leave the damp earth to climb shrubs and from there to drop on animals or man passing through the forest. Their bites are painless, but may be followed by ulcers. They may get into the nostrils.

They will even penetrate thick clothing in order to reach the skin.

## CHAPTER XXI

# THE ARACHNIDS

	Classification of the Arachnida				
Order	Family	Genus	Species		
	$\int$ Trombidiidae	Trombicula	{T. akamushi {T. irritans		
	Parasitidae	{Dermanyssus Liponyssus	D. gallinae L. bacoti		
	Tyroglyphidae	Tyroglyphus	T. farinae T. longior		
		(Glyciphagus	G. domesticus		
	Sarcoptidae	Sarcoptes	S. scabiei		
	Demodicidae	Demodex	D. folliculorum		
	Tarsonemidae	Pediculoides	P. ventricosus		
Acarina	Argasidae	Argas	A. persicus A. miniatus		
		Ornithodoros	∫O. savignyi ∤O. moubata		
	1	/ Ixodes	I. ricinus		
		Hyalomma	H. aegypticum		
		Rhipicephalus	R. bursa		
	Ixodidae	Dermacentor	{D. variabilis D. andersoni		
	{	Boophilus	B. annulatus		
	1	Amblyomma	A. hebraeum		
	1	Haemaphysalis	H. leachi		
		\Linguatula	L. serrata		
Linguatulida	Linguatulidae	Armillifer	A. armillatus		

The class Arachnida and the class Insecta belong to the phylum Arthropoda. This phylum contains a greater number of species than does any other phylum; in fact it exceeds in this respect all other phyla combined.

Other arthropods are the Myriapoda, or thousand-legged worms, and the Crustacea, to which belong the lobsters, crabs and water-fleas—important zoologically but of very slight importance medically. For Venomous Arthropods, see page 593.

The different classes of Arthropoda resemble the segmented worms but have as the point of distinction the possession of jointed appendages which proceed from the somites in pairs. Some of the pairs of limbs are for locomotion; at times, certain ones may be specialized for food taking.

The somites or divisions of the body have a chitinous exoskeleton. Respiration takes place through the medium of gills in the Crustacea and by tracheal tubes in the Myriapoda, Arachnida, and Insecta.

The Arachnida have no antennae whereas the Myriapoda and Insecta have a single pair of antennae, the former having numerous pairs of legs or jointed appendages whereas the latter have only three pairs of legs. The Arthropoda have an exoskeleton which is more or less unyielding from the deposit of chitin in the cuticle. This cuticle is not true skin but only a secretion of the epidermis.

Within this external skeleton there is a dorsal digestive system and a ventral nervous system.

Great importance of arthropods in medicine.—Members of this phylum are important not only because of certain immediate and direct effects of their activities, such as the action of poisons introduced by scorpions, spiders and ticks, or the painful and peace-disturbing attacks of various biting arachnids and insects, but in vastly greater degree in that among them are our most important transmitting agents of disease. The following is a list of the diseases transmitted by them.

Transmitted by arachnids.—Rocky Mountain spotted fever, tsut-sugamushi and other typhus-like diseases, tick-bite fever and the relapsing fevers of East and West Africa and of Panama.

Transmitted by insects.—Typhus fever, European and Indian relapsing fevers, trench fever, American and African trypanosomiasis, plague, tularaemia, filariasis and loaiasis, malaria, yellow fever, papataci fever, dengue, oriental sore and possibly other forms of leishmaniasis, together with certain helminthic infections (Dipylidium and Acanthocephala).

Transmitted through crustaceans.—Infections with Dracunculus medinensis and Diphyllobothrium latum through the medium of Cyclops, and paragonimiasis through certain species of crabs.

There are many arthropods which may accidentally bring about direct transference of disease, as with tabanid or stable flies which, following contamination of their biting parts with anthrax bacillus blood, might directly transfer the virus, when shortly afterward feeding on a man or animal. Many non-biting flies, in particular the house fly, and possibly cockroaches or other arthropods having access to our food or faeces, are important agents in the spread of typhoid, cholera, bacillary dysentery, and amoebic dysentery. The itch mite and the sand flea, as well as certain fly larvae, by burrowing into the skin or mucous membranes, are well recognized causes of disease. Fly larvae are also the causes of various intestinal myiases. Bacterial infections of mosquito or other insect wounds, by scratching, are frequently reported.

On pages 402 and 403 will be found a list of arthropodan diseases.

### THE ARACHNIDA

The Arachnida differ from the Insecta in having the head and thorax fused together. They also have four pairs of ambulatory appendages, whereas the insects have three pairs. The Arachnida never have compound eyes—eyes when present being simple. Of the two orders of Arachnida of interest medically the Acarina is far more important than the Linguatulida.

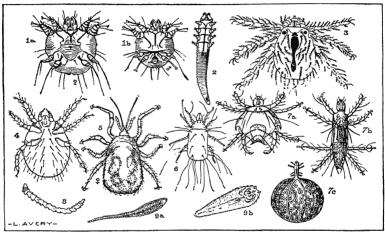


FIG. 129.—Arachnida exclusive of ticks. (1a) Sarcoptes scabiei, female; (1b) S. scabiei, male; (2) Demodex folliculorum; (3) Trombicula akamushi, hexapod larva (Kechani mite); (4) Trombidium holosericeum larva (Lephus); (5) Dermanyssus gallinae; (6) Tyroglyphus longior; (7a) Pediculoides ventricosus, male; (7b) P. ventricosus, young female; (7c) P. ventricosus, impregnated female; (8) Armillifer armillatus; (9a) Linguatula serrata, female; (9b) L. serrata, larva. Note: Figure not drawn to scale.

#### ACARINA

Of the acarines the most important are the mites and the ticks. The acarines do not show any separation of the abdomen from the cephalothorax. A hexapod larva develops from the egg; this is succeeded by an octopod nymph which differs from the adult in not having sexual organs.

In addition to the four pairs of legs in the fully developed acarine there are two other paired appendages; the chelicerae, in front of the mouth, and the pedipalps on either side of the mouth.

### TROMBIDIDAE (HARVEST MITES)

These generally have a soft, more or less hairy integument and are often brightly colored. The two eyes are often pedunculated and the chelicerae are lancet-shaped

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and the palps project beyond the rostrum as claw-like appendages. A tip-like appendage on the apical segment of the palps is characteristic. A very common and annoying member of this family is the hexapod larva of the *Trombicula irritans*. It is usually designated *Leptus autumnalis*. Popularly it is termed "harvest mite," "red bug" or "jigger." They are found in the fields in the autumn and attack both man and animals. They measure 150 $\mu$ , and climb up the feet and legs, their small size enabling them to enter through ordinary clothing. They do not penetrate the skin but soften the skin with a secretion from the hypopharynx. Serum from the host, hardening, makes a closed tube through which the larval mite feeds. After becoming engorged (one or two days) they drop off and moult on the ground, to become 8 legged nymphs. The adult mites do not attack man. The condition (itching and redness) produced is at times called autumnal erythema. There is a *Trombidium* (Trombicula) in Mexico which has a predilection for the skin of the eyelids, prepuce, and navel.

Trombicula akamushi.—It is called the Kedani mite. It is an orange-red larval mite about 400 by 200 $\mu$ . These mites are very hairy and live inside the ears of the field mice of the area of Japan in which the disease occurs. The adult forms live in the soil of the endemic area. They are about r mm. long by 0.5 mm. wide. The hexapod larva usually attaches itself to the axillary or groin regions and drops off after engorgement, leaving an eschar from which adenitis develops. The rickettsial infection, which is transmitted by the larval bite, is inherited from the parent—the larva not feeding a second time. The typhus-like disease is called tsutsugamushi and occurs in laborers harvesting hemp on the banks of certain Japanese rivers. It is often stated that the mice serve as reservoirs of virus. Other mite-transmitted rickettsias are associated with Trombicula—T. deliensis, in Sumatra and T. hirsti for Mossman Fever of Northern Australia.

### PARASITIDAE (CHICKEN MITES)

Of the Parasitidae, which generally have a hard leathery body and styliform piercing chelicerae, delicate five-jointed pedipalps and styliform hypostome, only the Dermanyssus gallinae is of interest. This mite infests chicken-houses and sucks the blood of the inmates. They will also attack man. Poultrymen may be troubled with a sort of eczema on the backs of the hands and forearms, similar to scabies, resulting from bites by these mites. They measure  $350 \times 650\mu$ . They have no eyes. The tropical rat mite, Liponyssus bacoti, will attack man and in some Southern states of the U. S. has been incriminated as a carrier of endemic typhus. The mite transmits the rickettsial disease to its eggs. These mites produce a dermatitis.

# Tyroglyphidae (Cheese Mites)

Mites of this family live on cheese, flour, dried fruits, etc. They are small, without eyes, and have a smooth skin and a cone-like appearance of the mouth parts which are largely formed by the chelate chelicerae. They are chiefly of importance because of their being occasionally found in urine, faeces, etc., and being striking objects, the question of pathogenicity arises. The Tyroglyphus longior has been associated with intestinal trouble (probably a coincidence, patient having eaten cheese containing these mites). Other species of Tyroglyphus have been held responsible for "vanillism" in those who work with vanilla pods, or for "copra itch," in copra workers.

Glyciphagi are found in sugar and are the cause of what is known as "grocer's itch." Rhizoglyphus parasiticus is reported to be the cause of an itch-like affection of the feet of coolies on tea plantations. To distinguish: The dorsum and legs of Glyciphagus are covered with plumose hairs; Tyroglyphus has both claws and suckers on tarsi, while Rhizoglyphus has only claws.

#### SARCOPTIDAE (ITCH MITES)

These are small eveless mites with a transversely striated cuticle. They live on the epidermis of man and various animals. The human itch mite, Sarcoptes scabiei, is an oval mite; the male is 250 × 150\mu, the female about 400 × 300\mu. Besides the difference in size, the male may be distinguished from the female by the fact that the third and fourth pairs of legs in the female have bristles whereas in the male the fourth pair has suckers (ambulacra). In these mites the rostrum is made up chiefly of chelate chelicerae with quite short three-jointed, rather adherent palpi. The female passes through four stages: (1) Larva; (2) nymph, resembles adult but has no sexual organs: (3) the pubescent female; (4) the egg-bearing female. A female becomes mature in about two weeks. The eggs, 140µ long, hatch out in four to five days. A pair of itch mites may produce 1,500,000 descendants in three months. The male does not burrow. Copulation takes place on the surface of the skin after which the male dies. Scabies is produced by the fertilized female who remains with her host, and not by the eggs, larvae, or pubescent female. The adult female burrows into the skin especially between the fingers, on the wrists and penis. In infants any part of the body may be affected. These tunnels are from 2 to 12 mm. long and tend to zigzag. They are dark gray, and at the entrance of the burrow the faeces accumulation makes a sort of minute dirty papule. A vesicular elevation marks the location of the mite at the blind end. Scratching obliterates these burrow lines. They are indistinct in those who bathe frequently (Gale des gens du monde). The tunnels have the egg-bearing female at the blind end; scattered all along are faeces, eggs and larvae, the eggs being next to the mother and the more mature young at the entrance to the gallery. The mites are more active when the patient's body is warm and relaxed, hence the nocturnal itching. A diagnosis can be made by demonstrating either eggs or larvae. It is rare to find males. Stokes (1936) stresses the occurrence of various allergic manifestations from sensitization to the protein of the itch mite. The urticarial response may continue weeks or even months after the lesions have cleared up. Treatment with sulphur preparations kills the adult and immature mites, but the eggs are not affected. Hence a second treatment after about 10 days is necessary to kill any young mites which may have developed from the eggs.

Different animals have different species of itch mites. The term "mange" is usually applied to infestations of domesticated animals.  $\Lambda$  serious mange of cats (Notoedres cati) may attack man, but the infestation in man quickly dies out (10 days).

## DEMODICIDAE (HAIR FOLLICLE MITES)

Demodex folliculorum.—This is a vermiform acarine about  $400\mu$  long, living head down, chiefly in the sebaceous glands of nose and forehead. The eggs are about  $75 \times 35\mu$ . A six-legged larva hatches from the egg and develops into an eight-legged adult after four moultings. Some of the cases of "blackheads" are due to this face mite, and from 50 to 90 per cent of human beings have been reported to harbor them (Ger-

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many). Statistics do not show greater frequency of mites in comedones than in normal glands. They do not seem to cause any ill effects in man, but a different species causes a severe mange in dogs.

# TARSONEMIDAE (LOUSE MITES)

This acarine family shows a complete dimorphism. The last two pairs of legs are widely separated from the front legs. The *Pediculvides ventricosus* is oval; the male is about 125  $\times$  75 $\mu$  and has claws at the extremities of the anterior and posterior pairs of legs; the two other pairs have hooklets and a sucking disc. The female is about twice as long but of the same breadth as the male, and has claws only on the anterior legs. The chelicerae are lancet-shaped and retractile. The large pedipalps are fused together anteriorly. The gravid female is like a ball and is about 1000 $\mu$  in diameter.

These mites are viviparous, a single female containing from 200 to 300 sexually mature mites. They live on various insect larvae found on cereals and other plants—particularly straw and cotton, and from handling, or sleeping on infested bedding material, man may contract a violent dermatitis, possibly covering the entire body. The eruption with wheals, papules and vesicles appears in about 15 hours—marked itching and burning and sometimes fever.

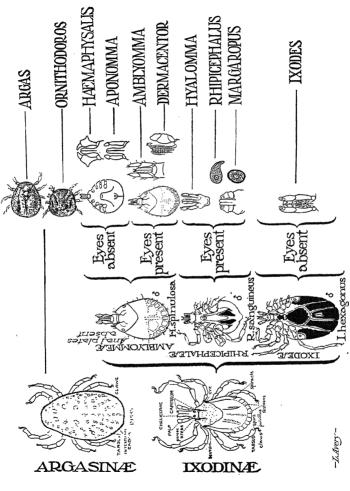
# IXODOIDEA (TICKS)

This superfamily is of great interest and importance medically.

Ticks differ from insects in having four pairs of legs, only two pairs of mouth parts, and no antennae. They differ from other acarines in having a median probe-shaped puncturing organ, the hypostome, which is beset with numerous teeth projecting backward, and in possessing stigmal plates. The head, or capitulum, or rostrum, is the part which projects anteriorly from the body. This carries the piercing parts which are the single hypostome or dart and a pair of piercing chitinous structures, the chelicerae, which lie above the hypostome. As a sheath for these delicate biting parts there is a segmented pair of palpi or pedipalps. The mouth is a slit between the chelicerae and hypostome.

When the tick reaches a host it first tears a hole in the skin with the chelicerae and then plunges the hypostome into the wound, continuing this process until the latter is completely embedded. The recurved teeth anchor this so firmly that if the tick is forcibly removed, either the hypostome is torn off and left in the wound or a fragment of skin is torn out with it.

There are two depressed pitted areas on the dorsal surface of the capitulum in the adult female known as porose areas. The stigmal plates are striking mosaic-like areas which are located just posterior to each hind leg in the Ixodidae and between the third and fourth legs in the Argasidae. The microscopic structure of the stigmal plates has been shown by Stiles to be of great value in differentiating the various species, especially of Dermacentor. The stigmal orifice, the opening of the tracheal system, is in the center. The Ixodidae have a scutum or shield-like chitinous structure on the dorsal surface. It covers almost the entire back of the tick in the male but only a small portion anteriorly in the female. The genital opening is toward the anterior part of the ventral surface. The anus, with anterior or posterior anal grooves, is near the posterior third of the venter. The legs have six segments, the coxa being flattened out on the surface of the body and the terminal tarsus ending with a pair of books and at



Rhipicephaleae. (Males having anal plates Includes Ixodeae. (Males clothed on all their ventral (Males without anal plates.) surface with anal plates in uneven numbers. Rostrum elongate. No eyes.) in pairs. Rostrum may be long or short. Eyes present.) Amblyommeae. Fig. 130.—Diagrammatic key to ticks, modified from Lahille.

times with a pulvillus. The nymph has stigmal plates but has no genital opening while the larva has neither genital apertures nor stigmal orifice.

Life History.—This varies with different ticks. That of Dermacentor may be taken as representative of the group. After the adults have succeeded in reaching a suitable host they engorge, mating occurs, and they drop to the ground. The males die at once. The females deposit their eggs in some protected place away from the host, and then they also die. From 2000 to 8000 eggs are deposited in the course of a month. (The number of eggs varies from one or two hundred in the Argasidae to 20,000 in certain of the Ixodidae.) After a period of development of a month or more, a small six-legged larva ("seed-tick") emerges. This crawls up a blade of grass and waits until it can attach itself to some passing animal. It then engorges and within a few days drops to the ground. Here it undergoes further development for several weeks, finally moulting and becoming an eight-legged nymph. This in turn climbs up a grass blade or a twig and awaits another passing animal (the second host). If fortunate enough to reach one, it engorges and again drops to the ground, and after several weeks development moults and becomes a mature adult. This must gain access to a third host in a similar manner to complete the cycle. A female tick may ingest 100 times its weight of blood.

The length of the life cycle varies greatly with the species and with weather conditions. In the case of D, andersoni it is over two years, the tick passing the first winter as an unfed nymph and the second winter as an unfed adult. In other species the cycle is completed in a season. Winter may be passed in the egg stage. The extraordinary capacity of the ticks to survive starvation compensates in part for the uncertainty and frequent delay in reaching a host. Larvae have survived 7 to 8 months and adults 3 to 5 years without food.

Many variations from this type of cycle have been observed. Among the Argasidae the adults often engorge and mate several times, on different hosts. In some Ixodidae the larvae and nymphs, and even the adults also, may complete their development on a single host, leaving only for oviposition ("two-host" or "one-host" ticks). In some species the males do not bite, and their mouth-parts are rudimentary.

### Classification of Ixodoidea

Family Argasidae.—Head concealed by body when viewed dorsally. No scutum. Stigmal plates between third and fourth legs. Adults have no suckers (pulvillus) beneath claws. Slight sexual dimorphism. Anus near middle of venter. Skin rough.

These ticks are chiefly parasites of birds, bats, etc., and occasionally of man, living and breeding in the nests or lair of their hosts, to which they have relatively ready access.

Genus Argas.—Body narrow in front. Margins thin and sharp. No eyes. Rostrum some distance behind anterior margin of body. A. persicus, (A. mianensis), the common fowl tick (Miana bug), transmits spirillosis of fowls. It has been suspected of transmitting a form of relapsing fever in Persia.

Genus Ornithodoros.—Body oval, margins rounded. Skin has many irregular tubercles. Rostrum even with anterior margin of body so that ends of palpi project slightly.

Ornithodoros moubata (the tampan) is common in central Africa from the Congo to Madagascar. The females are about 12 mm. long, they have a leathery cuticle covered with minute tubercles and flat rounded processes on the legs. There are no eyes. The ticks infest the native huts, particularly the rest houses along the routes of travel, hiding in crevices of the floors and walls during the day and coming out at night to bite the sleeping inmates. Both sexes bite man. It requires more than an hour to engorge. The females lay about 100 eggs. The larva develops to the nymph stage before leaving the egg, but the nymphs bite several times before maturity and the adults bite repeatedly.

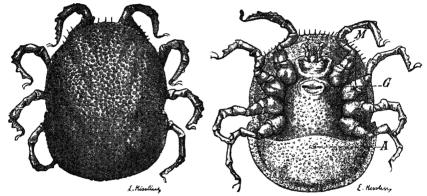


Fig. 131.—Ornithodoros moubata. (Murray from Doflein.)

This tick is the intermediate host of *Borrelia duttoni* (South African tick fever). Both adults and nymphs transmit the infection. The adult may transmit the infection through to egg to the young nymph, even to the third generation according to Möllers. The organisms become distributed throughout the tissues of the tick, including the salivary glands. The infection is conveyed by contamination of the wound with infected faeces or coxal fluid, and probably also directly by the bite.

Natives seem to suffer severely from tick fever in childhood but in adult life possess a sufficient degree of immunity so that the disease shows itself in a very mild form in those harboring spirochaetes. Ticks can be infected by these carriers. In some of the rest houses 50% of the ticks may be infected. While the tick does not tend to leave its habitation it may be transported in the bundles of native porters.

O. savingyi, which has two pairs of eyes, one pair near the base of the mouth parts and the other between the second and third coxae, is the intermediate host of B. duttoni in Ethiopia and southwestern Asia. Other strains of relapsing fever are transmitted by

O. erraticus in northern Africa and Spain; O. tholozani (possibly) in Iran; O. venezuelensis and O. talaje in tropical America; and O. turicata in central Texas.

Family Ixodidae.—Mouth parts project in front of body when viewed dorsally. Scutum present. Stigmal plates posterior to fourth pair of legs. Adults have suckers beneath claws. Skin finely striated.

Anus behind middle of venter. Sexual dimorphism marked. Male has well developed scutum; female has porose areas.

Section Ixodeae (Prostriata).—Transverse recurved preanal groove in female. Male has ventral surface covered with chitinous plates. No eyes. Genus Ixodes.

Ixodes has long rostrum with slender palpi—palpi narrow at base, leaving gap between them and hypostome. No festoons.

I. ricinus is the intermediate host of Babesia bovis, the cause of cattle fever of Europe. This and other species may cause tick paralysis.

Section Rhipicephalae (Metastriata).—Anal grooves behind the anus or absent in the female. Ventral surface of male without adapal plates (in *Dermacentor*, *Haemaphysalis*, *A ponomma* and *Amblyomma*) or with one or two pairs (in *Hyalomma*, *Rhipicephalus* and *Boophilus*). Marginal festoons present, more distinct in the males.

The more important genera can be distinguished as follows:

τ.	Palpi long and slender 2
	Palpi short 3
2.	Segments of palpi equal in length. Eyes present
	Second palpal segment much the longer 4
4.	Very ornate. Eyes present
	Not ornate. Eyes absent
3.	Eyes absent. Palpi conical. Rostrum broad. Not ornate
	Eyes present. Palpi not conical 5
5.	Anal grooves absent. Palpi have sharp transverse ridges. Not ornate Boophilus
	Anal grooves present, distinct
6.	Ornate. Square rostrum. Second and third joints of palpi as broad as long
	Dermacentor
	Not ornate. Rostrum hexagonal dorsally

Dermacentor andersoni (D. venustus) is a reddish brown tick with a dorsal shield marked by black and silvery-white lines. The male is about 2.5 by 4 mm. The young female is about 6 by 2.4 mm.; when replete, 15 by 9 mm. It occurs in the northwestern United States and British Columbia, where it is the vector of Rocky Mountain spotted fever. It transmits tularaemia and is a cause of tick paralysis. It appears to be gradually extending its range. The larvae and nymphs utilize small rodents as hosts, but the adult ticks require large mammals or man. The tick survives long periods of starvation. According to Cooley if the adult fails to find a host the first season it will hibernate and try again

the second year; if necessary, again the third year; and rarely may survive to try again the fourth year.

D. variabilis, the common dog tick of North America, is the vector of spotted fever in the eastern United States. It also transmits tularaemia. Other ticks which have been shown to convey rickettsial diseases are Amblyomma cajennense in Brazil, A. hebracum Rhipicephalus appendiculatus and Boophilus appendiculatus in South Africa, and Rhipicephalus sanguineus in Europe. Boophilus annulatus transmits Babesia bigemina, the cause of Texas fever of cattle. Larvae developing from eggs of female ticks which have fed on infected cattle transmit the disease. Several species of Rhipicephalus have been shown to transmit other piroplasmoses to various species of mammals. Haemaphysalis

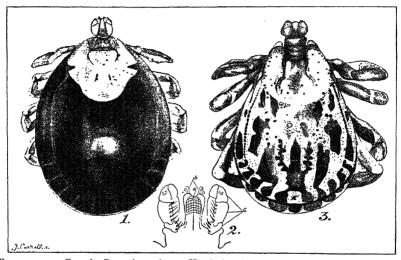


Fig. 132.—1. Female D. andersoni. 2. Head showing (a) hypostome, (b) chelicerae, (c) palps. 3. Male.

lepus palustris, the common rabbit tick, disseminates Rocky Mountain spotted fever and tularaemia among rodents, but rarely if ever conveys them directly to man.

It has been shown that several species of ticks will harbor the virus of yellow fever for from 4 to 28 days, and experimental transmission to monkeys has been reported to follow the bite of Ornithodorus rostratus, O. moubata, and Amblyomma cajennense 4 to 8 days after infection (Aragao, 1933). There is no evidence as yet of transmission by ticks under natural conditions.

Tick paralysis is an acute intoxication which is caused by the bites of rapidly engorging female ticks of certain species. It has been observed chiefly in sheep, dogs, and occasionally in children. It is most apt to occur

if the bite is about the head or neck. It is characterized by fever and an acute ascending paralysis (beginning in the legs) which might be confused with poliomyelitis. If the tick is discovered and removed promptly, recovery occurs in a few days. If this is not done, death may occur from paralysis of the respiration. It is believed to be caused by a venom secreted by the salivary glands of the tick during the period of rapid egg development. It has been reported chiefly from Oregon and British Columbia (where it is caused by *Dermacentor andersoni*), and also from portions of Europe, South Africa and Australia, where species of *Ixodes* have been incriminated.

### PENTASTOMIDA

These are degenerate, worm-like arthropods formerly classified with the Arachnida, although they are not closely related to any other group. The adults have elongated annulated bodies. They have two pairs of retractile mouth hooks. Otherwise there are no traces of legs, antennae or palpi in the adults. The species of medical interest are included in the family Linguatulidae (tongue worms).

Linguatula serrata (L. rhinaria) in the adult stage is usually a parasite of dogs or other carnivores (rarely of man), occurring in the nose or paranasal sinuses where it causes severe chronic inflammation and bleeding. The male is white, 2 cm. long; the female yellowish, 10 cm. long and has about 90 annular "segments." The eggs are thickwalled, contain a developed embryo, and are enclosed in a thin-walled bladder-like structure containing fluid. These are discharged with the nasal secretions. When ingested by a suitable intermediate host, usually a herbivore, occasionally man, a minute  $(75\mu)$  four-legged larva is liberated. This penetrates through the gut wall and reaches usually the liver or mesenteric glands where it develops, moulting several times and finally (after 6 months) encysts as a nymph. This is about 5 mm. long and resembles the adults except that there is a row of minute spines on the posterior margin of each ring. When the intermediate host is eaten the nymphs make their way to the sinuses and develop into adults.

The parasite is widely distributed but nowhere abundant. Human infection with adult parasites is very rare, but infection with the larvae is not uncommon in parts of Europe. Diagnosis intravitam is rarely possible. Little is known as to symptomology.

Armillifer armillatus is a parasite living in the trachea and lungs of pythons and other African snakes. The males are 3 to 5 cm. long with about 16 rings; the females 3 to 12 cm. long with about 20 rings. The eggs (80 to 100µ) contain a developed larva. They are passed with the bronchial secretions of the snakes and are ingested with contaminated food or water by monkeys, occasionally by various hervibores or by man. The larvae penetrate into the liver or other organs, undergo a protracted period of development (1 to 2 years) and finally encyst (nymph). When the intermediate host is caten by a snake the nymphs are liberated and penetrate into the lungs where they develop rapidly into adult forms. Many cases of human infection have been reported from west Africa, especially the Belgian Congo. A few cases of human infection have been reported from the Orient (with A. moniliformis) and two cases from America (possibly with Porocephulus crotali of rattle snakes).

# CHAPTER XXII

# THE INSECTS

# CLASSIFICATION OF THE CLASS INSECTA

Order	PAMILY SUB-FAMILY	TRIBE	Genus	Species
			Pediculus	P. humanus var. humanus
Anoplura	Pediculidae		Phthirus	P. humanus var. corporis P. pubis
	(			C. lectularius
Hemiptera	Cimicidæ		Cimex	C. rotundatus
	Reduviidae		Triatoma	{ T. megista T. sanguisuga
			( Pulex	P. irritans
	Pulicidae		Xenopsylla	X. cheopis
	Archaropsyllidae		Ctenocephalus	C. canis
Siphonaptera			Leptopsylla	C. felis L. musculi
	Histrichopsyllidae		∫ Ceratophyllus	C. fasciatus
	Dolichopsyllidae		Hoplopsyllus	H. anomalus
	Tungidae		Tunga	T. penetrans
			(Tabanus	T. glaucapis
			Haematopota	H. pluvialis
	Tabanidae		Pangonius	P. beckeri C. discalis
			Chrysops	C. dimidiatus
	Oscinidae		Hippelates	H. pallipes
			Glossina	(G. palpalis
			Stomoxys	C. morsitans S. calcitrans
			Musca	M. domestica
			Auchmeromyi	
	Muscidae		Fannia	F. canicularis
			Calliphora	C. vomitoria
			Lucilia	∫L. caesar
			1	L. sericata
			Phormia	P. regina
			Cochliomyia Chrysomyia	C. americana C. macellaria
			Cordylobia	C. anthropophaga
Diptera	Sarcoph-		Sarcophaga	S. carnaria
	agidae		Wohlfartia	W. vigil
	·		(Dermatobia	D. hominis
	Oestridae		Hypoderma	H. bovis
			Gastrophilus	G. nasalis
	Simuliidae		Simulium Eusimulium	S. damnosum E. metallicum
	Psychodidae		Phlebotomus	∫ P. papatasii
	Chironomidae		Culicoides	P. argentipes C. furens
	Cinronomidae	Sabethini	Wyeomyia	C. furens • W. smithii
	Culicidae Culicinae		√ Aedes	A. aegypti
		Culicini	Culex	C. quinquefasciatus
		Anophelini	Anopheles	A. maculipennis
	Corethrinae		Corethra	C. cinctipes

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# Insecta

The class Insecta has one pair of antennae, three pairs of mouth parts (the fused labium being considered as one pair), and three pairs of legs. They have three divisions of the body—head, thorax, and abdomen.

The head carries the antennae and mouth parts; the thorax, which is divided into the prothorax, mesothorax and metathorax, carries upon the ventral surface of each thoracic segment a pair of legs and on the dorsal surfaces of the two posterior segments a pair of wings. The abdomen does not support appendages. The air is supplied by means of tracheae—branching breathing tubes which have external openings or stigmata. The tracheae are stiffened by spiral chitinous bands. The Malpighian tubules are excretory organs of the alimentary system and excrete nitrogenous waste material. Insects have two pairs of wings, the second pair of which is frequently rudimentary and shows simply as knob-like projections. These are termed halteres or balancers. In some insects both pairs of wings are rudimentary, as in Siphonaptera.

When insects show metamorphosis voracious worm-like larvae hatch from eggs; these larvae are succeeded by a quiescent non-feeding encased pupa which finally develops into an imago or fully developed insect. An insect which does not present this developmental cycle shows incomplete metamorphosis. Of the class Insecta only the Anoplura, Hemiptera, Siphonaptera, and Diptera are of special importance.

# Anoplura (Siphunculata)

These are small dorso-ventrally flattened wingless insects not showing metamorphosis.

# Pediculidae

In this family there are no wings and there is no metamorphosis. They have simple eyes and 5 joints to the antennae. The legs are well developed and terminate in powerful claws. The young resemble the adults. The acorn-shaped eggs (nits) are deposited on hairs or clothing of the host.

Pediculus humanus var. humanus (Pediculus capitis).—The eggs, usually 60 in number, are deposited on the hairs of the head, the favorite region being back of the ears. They hatch out in about six days. The lice larvae on emergence closely resemble the adult and begin to feed shortly after hatching. They moult about every three days and become adults within ten days.

The adults vary in color according to the color of the hair of the host. The thorax is as broad as the abdomen. The male louse is smaller, is rounded off posteriorly and shows a dorsal aperture for a pointed penis; while the female is recognized by its larger size, 2 mm. in length, and by a deep notch at the apex of the last abdominal segment.

There seems to be a marked preference exhibited by lice for their own peculiar racial host. It has been suggested that this might account for certain peculiarities in infection where different races were living together and under similar conditions as to food and

environment, and yet only one race contracts the disease. The head louse has been found to harbor leprosy bacilli when living on a leper.

Pediculus humanus var. corporis (P. vestimenti).—This louse lives about the neck and trunk underclothing, being rarely found on the skin. The louse feeds about twice a day, deprivation of food killing the adult in nine days and the newly hatched louse in two days.

The female, under favorable temperature conditions (65°F.), begins to oviposit three or four days after reaching maturity, and thereafter, during her average life of four or five weeks, lays four or five eggs daily.

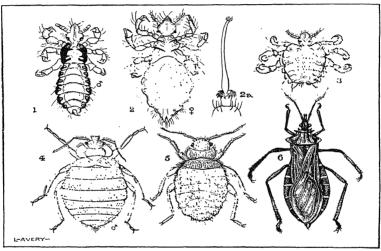


Fig. 133.—Anoplura and Hemiptera. 1. Pediculus humanus var. humanus. 2. Pediculus humanus var. corporis. 2a. Protruded rostrum of Pediculus. 3. Phthirus pubis. 4. Cimex lectularius. 5. C. rotundatus. 6. Triatoma megista.

The eggs, when on clothing next the body, hatch out in 7-10 days, and become mature in about two weeks. There is no grub stage as with the fleas.

As the eggs are usually deposited in inaccessible portions of the clothing, as in the seams, and since they remain viable there for more than a month, infested clothing should be steam-treated before being worn.

Analomy.—The body louse is somewhat larger than the head louse, and there is less marked festooning of the sides of the abdomen so that the segments are less well marked. The head is separated from the thorax by a narrow neck, there being but slight differentiation between the thorax and abdomen. The head is rather olive-shaped and more pointed anteriorly where is situated the mouth surrounded by a hook-bearing ring (the haustellum). The five-jointed antennae are attached to the side of the head. The three stylets are inside the mouth and are long and sharp. They can be protruded through the mouth orifice and when apposed make a tube through which the secre-

tions from the salivary glands empty. These needle-like stylets make the wound which causes the blood to flow. The blood is sucked up by the pharynx which lies above the stylets and has muscular walls making it a pumping organ. From this a narrow oesophagus leads into the long prominent stomach which terminates in a narrow S-shaped hind-gut. The tracheal system (respiratory) opens along the sides of thorax and abdomen in round openings or spiracles. These can be closed by oils, thus asphyxiating the louse. The female has a pouch-like opening beneath the hind-gut which leads to the oviduct which connects with two ovaries.

The eggs receive the cement material in the oviduct.

As the body and head louse differ more in habit or location than in structure and will interbreed readily, they are generally regarded as varieties of the same species, i.e., *Pediculus* 

humanus, varieties humanus and corporis.

The body louse (and probably the head louse) has been shown to transmit epidemic typhus fever and trench fever. Mackie (in India) and Nicolle (in northern Africa) showed that it is a vector of some strains of relapsing fever (in Europe, India, China, northern Africa and North America). Whereas the louse can transmit typhus and trench fever by biting, the spirochaetes are introduced only by scratching and rubbing the infective body fluids of the louse into the wound. Typhus and trench fever are usually introduced in the same way, either the crushed body of the louse or especially the faces being infectious.



FIG. 134.—Female Pediculus humanus, var. corporis. (Schamberg, after Kucchenmeister.)

of the louse or especially the faeces being infectious. These viruses apparently undergo some developmental cycle in the louse, since the louse is incapable of transmitting these diseases until several days have elapsed after the infecting feeding: typhus fever, 8 to 10 days (the faeces may be infectious after 3 or 4 days); relapsing fever, 4 days; trench fever, faeces infective after 7 days.

Phthirus pubis.—This louse is popularly known as the crab louse. The female is little more than  $\frac{1}{25}$  inch in length, and the male a trifle less. They are almost square. The second and third pair of legs are supplied with formidable hooks. They have a preference for the white race and live about the pubic region. The female lays about a dozen eggs, which hatch out in about a week. It is not known to be a vector of any infection.

The rat louse, *Polyplux spinulosus*, is important as a vector of endemic typhus among rats. The rabbit louse, *Haemodipsus ventricosus*, was shown by Francis to be a vector of tularaemia among rabbits. The dog louse (*Trichodectes canis*) is one intermediate host of *Dipylidium*.

# HEMIPTERA (RHYNCHOTA)

The Hemiptera or bugs are insects possessing mouth-parts modified for sucking in which the lower lip or labium or beak, having 3 to 4 seg-

ments, has its edges curved to form a groove. Within this groove are the biting parts—the bristle-like mandibles and maxillae.

The former are doubly grooved on their internal surfaces and thus when apposed form two tubes, one for injection of saliva and the other for suction of juice or blood. The maxillae support the mandibles. When in repose the beak or rostrum is bent back under the head or thorax. The beak is covered by the labrum only at its base, thus differing from the Diptera in which the labrum goes into formation of the sucking tube.

Bugs have no palpi. They have two pairs of wings which in some genera, however, are rudimentary. The metamorphosis in this order is incomplete.

### Cimicidae

These have a flattened body, a three-jointed rostrum, and four-jointed antennae. Their wings are atrophied.

Cimex lectularius (Acanthia lectularia).—This is the cosmopolitan bedbug or chinch. It measures about 15 by 16 inch (5 by 3 mm.). It is brownish-red in color. The most conspicuous feature of the bedbug is the long proboscis continuous with the dorsal integument of the head and tucked under the ventral surface. In biting the proboscis is straightened out and 4 piercing stylets are protruded to puncture the skin. There are two prominent eyes and two four-jointed antennae. The prothorax is flattened at the side. There are eight abdominal segments. The bedbug lives in cracks and crevices, especially about beds. It is said they can migrate from house to house. It is certain that they are frequently transferred with wash clothes. They have a penetrating odor when crushed. The female deposits about 50 eggs at a time in cracks and in ten days they hatch out into larvae which pass insensibly into adults by a series of five moultings during a period of 2 or 3 months. The depositing of eggs occurs about four times a year. The larvae bite as well as the adults. The average period of active life is probably from 3 to 6 months, but in a cool place they may survive a year without food. They will also bite other animals and occasionally infest chicken coops and laboratory animal cages. They are easily killed by moderately high temperatures (110° to 120°F.).

Cimex rotundatus (A. rotundata).—In India the C. rotundatus is the one encountered. It is dark mahogany in color, has a smaller head, narrower abdomen, thick rounded prothoracic borders and is more densely covered with hairs than C. lectularius.

Related species of bugs parasitic on other animals, particularly bats and birds, occasionally attack man, but they rarely establish themselves in human habitations.

Relation to disease.—The bedbug has been suspected of being a transmitter of many different infections, but there is no convincing evidence that it plays a really important part in the natural transmission of any of them. Francis showed that it is readily infected with P. tularensis and transmits the infection by biting (mice). It can be infected with plague bacilli, and animals can be infected by rubbing the crushed bug or its faeces into the skin, but not by a bite. The same appears to be true of Borrelia recurrentis, Leptospira ictero-haemorrhagiae and Trypanosoma

cruzi. It probably plays no part in the spread of typhus fever or Leishmania infections. The ordinary pathogenic bacteria do not multiply in the bug because of the bactericidal action of the contents of its digestive tract.

### Reduviidae

These hemiptera are popularly known as assassin bugs, corsairs, or kissing bugs. They have a long narrow head and a distinct neck. They are vigorous fliers and runners. The majority are predactious, feeding on other insects, but if disturbed may inflict very painful bites on man. A few have become blood-sucking parasites, and are of great medical importance because several species are hosts of the South American trypanosome, T. cruzi. The parasite undergoes a regular cycle of development in the bug and is transmitted by it. (See section on Trypanosomiasis.) These species are nocturnal biters, and their bites are relatively painless. They are common in tropical America.

Triatoma megista (Conorhinus megistus) is the important vector in Brazil. It is called "barbeiro" because of its preference for biting the face. It is black with red markings on the wings, abdomen and prothorax. The antennae (in this genus) are inserted midway between the eyes and the point of the head. The bugs live in the native huts, hiding in cracks during the day and feeding on the inmates at night. Its habits are like those of the bed bug. The wingless larvae (which also bite) hatch out of the eggs in about a month and attain maturity in about a year. The infection is spread among the bugs by their habit, when opportunity offers, of sucking blood from the distended abdomens of their companions, and (in Rhodnius) by the coprophagous habits of the larvae.

Triatoma infestans replaces the preceding species as a vector from southern Brazil southward and westward through Chili, Bolivia and Argentina. Other species naturally infected include T. braziliensis, T. dimidiata, T. geniculata, T. rubrovaria and T. sordida.

Rhodnius prolixus (and R. pictipes) replaces Triatoma as the principal vector in northern South America. It is brown with yellowish markings. The antennae in this genus are inserted near the extremity of the head.

Several species occur in the southwestern United States, including:

Triatoma sanguisuga (Conorhinus sanguisugus) the Texas or Mexican "bedbug." It formerly preyed on the common bedbug, but having acquired a taste for human blood through Cimex or Acanthia it now prefers man. It is nearly an inch long, dark brown in color, with a long flat narrow head and a short thick rostrum. It is spreading northward. It has been infected with T. cruzi experimentally.

#### SIPHONAPTERA

These are laterally flattened, markedly chitinized, wingless insects which undergo a complete metamorphosis.

This order is divided by Dalla Torre into two sub-orders—(1) the Fracticipita which contains the family Histrichopsyllidae of which the genus Leptopsylla is of medical interest and (2) the Integricipita containing the following families and genera of medical interest. Pulicidae—genera Pulex and Xenopsylla; Archaropsyllidae—genus Clenocephalus; Dolichopsyllidae—genera Ceratophyllus and Hoplopsyllus; Tungidae—genus Tunga.

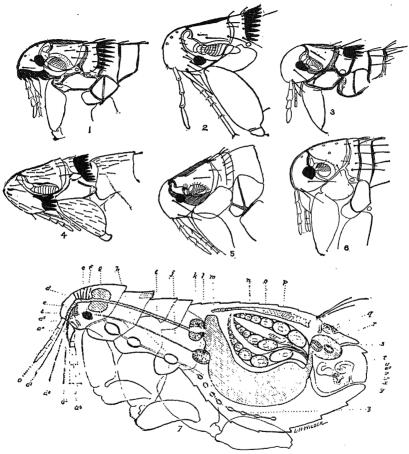


FIG. 135.—I. Ctenocephalus felix. 2. Ceratophyllus fasciatus. 3. Hoplopsyllus anomalus. 4. Leptopsylla musculi. 5. Xenopsylla cheopis. 6. Pulex irritans. 7. Internal anatomy of flea. (After Fox.) (a) Maxillary palpus; (a-1) epipharynx; (a-2) mandible; (a-3) labial palpi; (a-4) maxillae; (a-5) basal elements of rostrum and mandibles; (b) salivary pump; (c) hypopharynx; (d) aspiratory pharynx; (e) muscles of the aspiratory pharynx; (f) eye; (g) cesophageal ganglia (brain); (h) thoracic ganglia; (i) oesophagus; (j) salivary duct; (k) gizzard; (l) salivary gland; (m) stomach; (n) aorta; (o) ovaries; (p) Malpighian tubules; (q) pygidium; (r) rectum showing rectal glands; (s) anus; (l) intestines; (u) bursa copulatrix; (u-1) ductus obturatorius (blind duct); (v) receptaculum seminis or spermatheca; (w) ducts of spermatheca; (x) vagina; (y) uterus; (z) abdominal ganglia.

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In the Tungidae family, which will be discussed separately, the abdomen of the female becomes enormously distended with eggs, and she remains fixed in the burrow she has made under the skin, whereas in all other families the female remains practically unchanged with freedom of movement after fecundation.

The body of the flea is flattened laterally. They may or may not have eyes and rows of conspicuous stout spines called combs which are of importance in classification. The puncturing apparatus of the flea consists of a pointed epipharynx and two distally serrated mandibles. These chitinous biting parts are contained in the labium which divides distally into two labial palps. The maxillae are conspicuous triangular structures and, projecting farthest anteriorly, are the conspicuous four-jointed maxillary palps, often mistaken for antennae. By the apposition of the internally grooved mandibles to the epipharynx a tube is formed through which the blood is sucked up. The antennae are inconspicuous and are in close apposition to the sides of the head, behind the eyes, and can only be well made out with a lens. Fleas have three pairs of legs, and the male can be distinguished from the female by its smaller size and the conspicuous coiled-up spring-like penis within the abdomen. The female has a conspicuous gourd-like spermatheca which varies in shape in different species. A very prominent structure is a pitted plate in the ninth abdominal segment (pygidium). Of importance in classification are prominent bristles originating from the seventh abdominal segment and projecting over the pygidium. These bristles vary in number and are known as antipygidial bristles.

The eggs are laid in the dust of floors, under rugs, or loosely on the hair or in the nests of their hosts. After 3 or 4 days a bristled worm-like larva emerges from the egg. It has 14 segments and a distinct head with biting mandibles. The larvae do not suck blood but feed on any sort of organic material surrounding them. In this way, probably, they may ingest the eggs of Dipylidium caninum or Hymenolepis diminuta, the larval stages of which develop later in the adult flea. Should such an infected flea be taken into the mouth (of a dog, rat or child), tapeworm infection results. Some species require blood, which they get from the semi-digested faeces of the adult fleas. After one to two weeks or more the larva forms a cocoon and develops into a nymph with three pairs of legs. The adult flea emerges after about three weeks. The whole cycle usually takes from one to three months. The adult fleas under favorable conditions may live from one to two years, but they die quickly in hot dry climates. If cool and moist they may live for several months without feeding.

KEY TO FLEAS COMMONLY FOUND ON RATS AND CALIFORNIA GROUND SOUIRRELS

### A. With combs.

- 1. Eves present.
  - (a) Combs along inferior border of head and on prothorax. Ctenocephalus canis and C. felis (Ctenocephalides canis, C. felis).
  - (b) Combs only on prothorax.
    - Rostrum extending to trochanters.
       Prothoracic comb of about 18 spines.
       Ceratophyllus fasciatus (Nosopsyllus fusciatus).
    - (2) Rostrum extending well beyond the trochanters. Prothoracic comb of about 18 spines. Ceratophyllus acutus (Diamanus montanus).

- (3) Rostrum scarcely reaching half the distance to the anterior coxac. Prothoracic comb of about 9 spines.
- Hoplopsyllus anomalus.
- 2. Eyes absent.
  - (a) Collar of combs on prothorax and four short ones along inferior border of head. Leptopsylla musculi (Ctenopsylla musculi) (C. segnis).
- B. Without combs.
  - (a) Ocular bristle arises near upper anterior margin of eye. A line between this and the oral bristle approximately vertical. Two bristles posterior to antennae. A vertical ridge-like thickening on mesonotum. Xenopsylla cheopis (Xenopsylla pallida?). Formerly Pulex cheopis.
  - (b) Ocular bristle arises near lower anterior margin of eye. A line between this and the oral bristle approximately horizontal. One bristle posterior to antennae. Pulex irritans.

The common human flea of Europe is Pulex irritans, which is cosmopolitan in temporate regions; of the United States, P. irritans in California; Ctenocephalus canis, the dog flea, and C. felis, the cat flea, in the eastern states. The species primarily responsible for the transmission of plague is Xenopsylla cheopis, the Indian rat flea, the commonest rat flea in the warmer regions throughout the world. Originally a parasite of the black (Indian) rat, Rattus rattus, it now equally infests the brown rat (R. norvegicus) in warm climates. It resembles P. irritans but is more yellow than brown in color. It also has a greater number of bristles on the head. Ceratophyllus fasciatus is the common rat flea of Europe and the United States. Ctenocephalus canis and felis, Leptopsylla musculi, and Pulex irritans have also been found frequently on both Rattus rattus and R. norvegicus.

Relation to Disease.—As a result of the convincing experiments of the Indian Plague Commission, their rôle in the transmission of plague was absolutely established. It is by the bite of *Xenopsylla cheopis* that plague is chiefly transmitted from rat to rat, and in bubonic and septicaemic plague it is apparently the intermediary in human infection. Any species of flea which lives on the rat is capable of transmitting plague, as would also *Pulex irritans* if fed on the blood of a human case of septicaemic plague.

The average capacity of a flea's stomach is about 0.5 cu. mm. so that with a rat dying with speticaemic plague and with possibly 100,000,000 bacilli to 1 cc. of blood the flea would take in about 5000 bacilli. Furthermore these multiply in the alimentary canal so that the digested blood teams with bacilli when reaching the anus of the flea. The plague bacilli are passed out with the faeces, and these being rubbed into the puncture of the flea bite bring about infection. Regurgitation, as result of obstruction by masses of plague bacilli in the oesophagus, causes injection of plague bacilli into rat or man in the act of biting. This is more important than inoculation with the faeces.

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Trypanosoma lewisi (and possibly other rodent trypanosomes) also is transmitted by fleas, either Pulex irritans or Ctenocephalus canis. The trypanosome undergoes development in the flea, the infecting material is in the faeces of the flea, and transmission occurs by the licking on the part of the rat of faeces from an infected flea. The infection has no connection with the puncture wound of the flea as is the case with plague.

The rat fleas (X. cheopis and C. fasciatus) also serve as vectors of endemic typhus (Rickettsia mooseri), conveying the organisms from rat to rat and rat to man. Fleas may convey tularaemia from rodent to rodent but are not known to have infected man. The evidence at present indicates that they are not concerned with the transmission of the trypanosome diseases of man or the larger mammals, or of Leishmania.

Rat Hosts.—To distinguish R. norvegicus from R. rattus we have in the former (1) ears which barely reach the eyes when laid forward and (2) tail rather shorter than length of head and body together (only 89% of length of head and body together). With R. rattus the tail is longer than the head and body together (25% longer) and the extended ear covers or reaches beyond the middle of the eye. R. rattus has a sharper nose, longer and more delicate tail and thinner ears than R. norvegicus (formerly R. decumanus). R. alexandrinus is a variety of R. rattus.

In California the ground squirrel, Citellus beechyi, acts as a reservoir of plague, and has as flea transmitters Hoplopsyllus anomalus and Ceratophyllus acutus. Other important reservoirs of plague among wild rodents are marmots in Mongolia, ground squirrels in southeastern Russia and central Asia, field rats in Java, and gerbilles and karoo rats in South Africa.

Tungidae.—Belonging to the family Tungidae, the Tunga penetrans (Dermatophilus penetrans) is of great importance in tropical countries. It is known as the chigoe, nigua, or jigger. The male and virgin female are relatively unimportant as they do not penetrate the skin but act as ordinary fleas. The female, which when unimpregnated is only about  $\frac{1}{24}$  inch long, when impregnated bores its way into the skin of man, especially about the toes, soles of the feet or finger-nails, and in the chosen site develops enormously, becoming as large as a small pea. This enlargement takes place in the second and third abdominal segments which are packed with eggs measuring about  $400\mu$  long and numbering about roo. Clinically, a small black spot in the center of a tense, rather pale area is characteristic. The metamorphosis is similar to that of the flea. Tunga can be differentiated from the flea by the proportionately larger head, and especially by the fact that the head has the shape of the head of a fish, distinctly pointed. With the fleas the lower border of the head comes out in a straight line to join the curve of the upper part. In the Tunga lower and upper border of head are both curved.

### DIPTERA

The insects of the order Diptera are of great importance medically, either because of the direct irritation of their bites, because of their transmitting disease directly, as does the common house fly typhoid fever, or

because of acting as intermediate or definitive hosts for various parasites. They are characterized by mouth parts formed for puncturing, sucking, or licking. They present a complete metamorphosis, larva, pupa, and

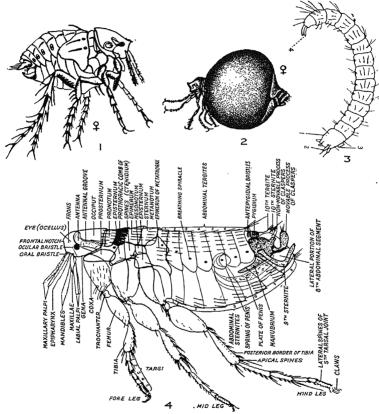


FIG. 136.—1-2. Tunga penetrans. (1) Male; (2) egg-distended female. 3. Flea larva highly magnified. (1) Head; (2) antennae; (3) egg-breaker; (4) caudal stylets. (From Byam and Archibald.) 4. External anatomy of a flea. (After Fox.)

imago. As a rule, the Diptera have one distinct pair of wings, the second pair being rudimentary (halteres).

The anterior portion of the head which lies below the origin of the antennae is the face, and on each side of the face we have the cheeks which should be studied as to presence or abundance of hairs. The antennae which separate the from from the face are of great importance in classification. In the Muscidae the appearance of a feathery structure, projecting from the terminal segment of the antennae, and called the *arista*, is important. This may be bare or feathered, and the feathering may be only on one side or of one part.

The males of flies in which the two compound eyes come together above the antennae are referred to as holoptic; if more or less widely separated, as dichoptic. Ocelli are single eyes, usually three in number, and, when present, situated in the triangular space between the compound eyes in the frons (the space separating the compound eyes).

In studying the biting flies it is very important to recognize the anterior, small, or mid-cross vein in the wings. This short transverse rib or vein is the key to wing venation. Beneath it is the discal cell, and it bounds the first posterior cell internally or basally. The fourth longitudinal vein, which touches the bottom of the mid-cross vein, is of particular importance as it gives different shapes to the first posterior cell as it runs along the lower border of this cell. The closed-in discal cell is below the fourth longitudinal vein. The character of the antennae should also be noted carefully. The study of the bristles about head, thorax, and abdomen (chaetotaxy) is more difficult. Anyone taking up the study of flies should note carefully the wings, etc., of Musca domestica. By putting a few house flies on moist horse manure in a gauze-covered bottle the entire metamorphosis may be observed.

#### CLASSIFICATION OF DIPTERA

- I. Suborder Orthorrhapha. The larvae have a well-differentiated head. Pupa naked. Imago escapes from the pupal case through a T-shaped break on the dorsum near the anterior end. No frontal lunule. Wing venation simple.
  - a. Series 1. Nematocera. Midge-like insects with long many-jointed (8 to 16) antennae and usually long slender palpi of one to five segments. Anal cell not narrowed toward the wing margin.
    - α. Marginal vein not continued beyond the tip of the wing.
      - Simuliidae. (Buffalo gnats, black flies). Antennae shorter than the thorax, with 11 segments, not plumose. Wings broad, without scales or hairs.
      - Chironomidae: (Midges). Antennae longer than the thorax, bushy, with 14 segments. Wings narrower, median vein forked. Wings bear setae, no scales.
    - β. Marginal vein extends entirely around the wing. Second and fourth longitudinal veins forked.
      - Psychodidae (Moth midges, sand flies). Wings and body thickly covered with coarse hairs. Wings ovate or pointed.
      - Culicidae (Mosquitoes). Without these characters. Wings with rounded tips. Posterior margin and veins of wings fringed with scales. Mouth parts long, slender, adapted for biting.
  - b. Series 2: Brachycera. The antennae are short, composed of only two or three simple joints, with or without a style or arista. The palps are nearly always short

and never more than two-jointed. Anal cell closed or narrowed toward the margin of the wing.

Tabanidae (Horse flies). Third joint of antennae complex. Costal vein surrounds the wing.

II. Suborder Cyclorrhapha. Head of larvae not differentiated. Pupa enclosed in last larval skin. Imago escapes through an anterior circular opening in the puparium

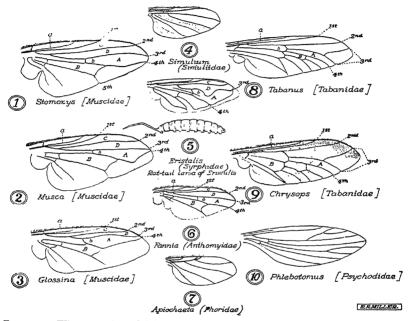


Fig. 137.—Wing venation of Diptera. A, First posterior cell; B, discal cell; b, midcross vein; a, auxiliary vein; C, marginal cell; D, submarginal cell.

produced by the ptilinum. (This is an inflatable organ projecting just above the root of the antennae and is retracted as soon as the image escapes, leaving behind typical scars, the frontal lunule and the frontal or ptilinal suture (see Fig. 138). Wing venation more complex.

- a. Series 1. Aschiza. Ptilinum small, frontal suture absent.
  Family Syrphidae (Hover flies). Anal cell ends in an acute angle, reaching nearly to the wing margin. A spurious "vein" between the third and fourth longitudinal veins. (Mostly large, brightly colored flies. A few cases of human intestinal myiasis have been reported due to the "rat-tailed" larvae of Eristalis.)
- b. Series 2. Schizophora. Ptilinum large; frontal suture and lunule marked, causing retraction of front of head. Head freely movable.

### α. Pupipara.

Family Hippoboscidae. Blood-sucking ectoparasites of birds and mammals with tough leathery bodies, poorly developed wings and indistinctly segmented abdomens. The larvae are almost completely developed at birth. (A few species are vectors of disease; e.g., Melophagus ovinus, the sheep "tick" or ked, conveys sheep trypanosomiasis, and Lynchia maura is the definitive host of Haemoproleus columbae of pigeons. None are known to convey human infection.)

β. Myodaria. Without these characters.

Acalypterate myodaria have no scales covering the halteres.

Oscinidae (Chloropidae), the eye flies.

(Also includes the cheese fly, *Piophila casei* and other small flies, chiefly of minor medical interest.)

Calypterate Myodaria have large scales (squamae) covering and concealing the halteres. (Includes a very large number of species, many of great medical importance.)

- 1. Oestridae (Bot flies). Mouth opening small, mouth parts rudimentary.
- Sarcophagidae (Flesh flies). Mouth parts normal, adapted for sucking. Arista of antennae plumose on proximal half only. Hypopleural bristles present.
- Muscidae (Large family). Mouth parts adapted for sucking or biting. Arista of antennae plumose to the tip.

#### Tabanidae

This family includes the horseflies, gadflies, breeze flies or green-headed flies. It is the most numerous family of the Diptera—there being nearly 2500 species. The females are blood-suckers; the males live on flowers and plant juices. The eyes are usually large, very brilliant in color, and in the male make up the greater part of the head.

They belong to the suborder Orthorrhapha and in the group of short-antennae flies (Brachycera). The wings are large and encircled by the costal vein. The third longitudinal vein is forked. The fourth longitudinal vein breaks up three times thus enclosing the discal cell. Five posterior cells are always present. The squamae are large.

The antennae consist of three segments, the third of which is compound. No arista. The mouth parts are complete in the female. The epipharynx is tube-like, the hypopharynx has a groove and both are awl-shaped. The paired maxillae are serrated and the mandibles lancet-like. They have rather coarse maxillary palps. The labellae are prominent at the extremity of the fleshy labium. In the male the mandibles are atrophied. The Tabanidae are thick-set flies and rarely show color. The body of the larva has eleven segments and a small but distinct head. The eggs are deposited in masses on the leaves or stems of plants about marshy places. The larva is carnivorous. Of the numerous genera of Tabanidae the more important are:

Tabanus.—No ocelli. No spurs at tips of hind tibiae. The last (third) antennal segment is composed of five parts and shows a crescentic notch. Wings clear or smoky, not spotted, do not overlap. A huge genus (over 1000 species), world wide in distribution, chiefly large powerful fliers which bite viciously both man and animals. T. glauca pis is the intermediate host of Trypanosoma theileri, a non-pathogenic parasite

of cattle (Europe). Tabanids are suspected of being the vector of *Leishmania* in the forested regions of southern Brazil and Paraguay.

Haematopota.—There is no crescentic antennal notch, and the third antennal segment is composed of four parts. The wings overlap and show scroll-like markings. The abdomen is narrower than in Tabanus. No ocelli. Common in Africa and the Orient. The brimp, one of the Haematopota, bites man severely.

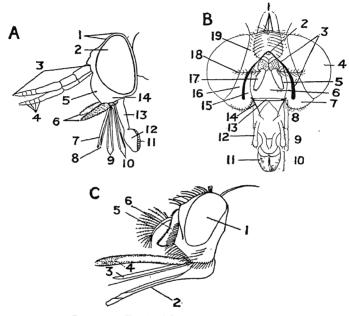


Fig. 138.—Heads of flies (semidiagrammatic).

A, Chrysops discalis. (1) Ocelli; (2) compound eye; (3) antennae; (4) annuli; (5) clypeus; (6) palpi; (7) labrum-epipharynx; (8) hypopharynx; (9) mandibles; (10) maxillae; (11) pseudotracheal membrane; (12) labellae; (13) labium; (14) gena.

B, Muscoidean fly. (1) Ocelli; (2) lunula; (3) ptilinal suture; (4) compound eyes; (5) antennal grooves; (6) clypeus; (7) genae or cheeks; (8) rostrum; (9) haustellum; (10) labellae; (11) pseudotracheal membrane; (12) palpi; (13) epistoma; (14) oral vibrissae; (15) facialia; (16) parafacials; (17) antennae; (18) arista; (19) parafrontals. (After Fox.)

C, Glossina sp. (1) Eye; (2) labium; (3) labrum; (4) palp; (5) arista; (6) antenna.

Pangonius is characterized by a very long, slender and more or less horizontal proboscis. The antennae are small. The third segment is composed of seven or eight parts. Wings clear or smoky.

Chrysops has three ocelli. The antennae are especially long and slender and have three segments, the last one of which is composed of five parts. The wings are widely

separated and have a dark band along the anterior margin and a broad dark cross band one third removed from the tip. They inflict painful bites. There are many species, distributed throughout the world. C. dimidiata and C. silacea serve as intermediate hosts of Loa loa (tropical Africa).

Many species of tabanids may act as mechanical conveyors of infection although the organisms do not develop or multiply in the fly. This has been proved in the case of anthrax, e.g., and is of practical importance in numerous trypanosome infections, including *T. evansi*, *T. brucei*, *T. equinum* and *T. equiperdum*. It is essential that the first feeding of the fly on an infected animal be interrupted and that the fly bite a second animal within about ten minutes.

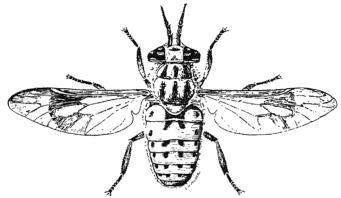


FIG. 139.—Chrysops discalis, showing the characteristic non-pigmented discal cell whence is derived its name.

Chrysops discalis, the western deer fly, was shown by Francis and Mayne (1921) to convey Pasturella tularensis in this way, both to man and animals. The fly remains infective for at least two weeks.

#### Oscinidae

The "eye" flies which belong to the acalypterate Myodaria are small hairless flies about 2 mm. long. They usually occur in swarms and cause annoyance by flying into the eyes or ears or crawling over open wounds or ulcers to lap up secretions. Their habits are similar to those of the house fly, including that of regurgitating the contents of the oesophageal diverticulum while feeding ("vomit drop"). Hippelates pusio (in California and elsewhere) has been shown to spread "pink eye" and other infectious forms of conjunctivitis in this way. Krumm and Turner (1936) in Jamaica have demonstrated that H. pallipes, after feeding on open yaws ulcers, contains many motile spirochaetes in the oesophagus for several hours, and they have produced yaws lesions in rabbits by allowing infected flies to feed on open skin wounds.

## Muscoidea

Under this heading we may group the Muscidae, Sarcophagidae and Oestridae which are calyptrate Schizophora.

## Muscidae

In the Muscidae the antennae hang down in front of the head in three segments and have an arista plumose to the tip on one or both sides. The first posterior cell is narrowed, due to bending up of 4th vein. There are no bristles on abdomen except at tip.

Adult Muscid Flies That Do Not Suck Blood.—In a majority of the Muscidae the proboscis is relatively short and stout with fleshy labella, and is adapted to licking or rasping but not to biting. (The maxillae and mandibles are atrophied, even in the biters.) These flies are important medically because, as a result of their filthy habits of eating and breeding, they often convey infection mechanically, and because the larvae of some of them cause myiasis. The more important genera are given in the following key:

Hypopleural bristles absent.
Sixth vein short; fourth vein straight
Sixth vein longer; fourth vein angled at the distal end. Color opaque greyish-
black
Hypopleural bristles present.
Color greenish or bluish black.
Basal section of first vein ciliated.
Face yellow; one posthumeral bristle(Chrysomyia) Cochleomyia
Face black with black hairs; two posthumeral bristles. Prothoracic spiracle
light orange
Basal section of first vein bare.
Upper surface of lower squamae bare
Upper surface of lower squamae pilose
Color yellowish or reddish, non-metallic.
Eyes of male approximated. Five or more ranges of setae on orbitCordylobia
Eyes widely separated. One seta on each orbit

Musca domestica.—The common housefly,  $Musca\ domestica$ , is the best example of this family.

The arista is feathered both dorsally and ventrally with straight hairs. The fourth longitudinal vein bends forward in a rather sharp angle as compared with Stomonys, the first posterior cell of the latter having rather a fusiform appearance. The eyes are close together in the male, far apart in the female. In contrast to the other flies in this group Musca has no large bristle on the inner surface of tibia of the middle legs. The female lays about 125 eggs in a heap, preferably in fermenting horse manure. The larva comes

out in about thirty-six hours. Very characteristic are the stigmata decorating the blune posterior ends. (See illustration, page 560.)

The larval stage lasts seven to ter days, and then the larva shrinks but remains surrounded by its old skin, termed puparium, which forms the covering for the barrel-shaped pupal stage. This lasts about three days when the adult fly emerges. This is termed a "coarctate" pupa. This fly is incapable of biting, the piercing organs being fused with the labium, but may transmit disease directly, carrying infectious material from the source, as faeces, to the food about to be ingested. Their rôle in typhoid fever is one of importance. By reason of its hairy sticky legs, habits of frequent defecation and constant regurgitation of the contents of the oesophagus, the housefly is an important agent in the spread of cholera, dysentery, infantile diarrhoeas and tropical ophthalmias as well as typhoid.

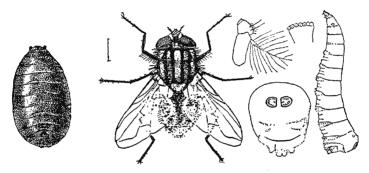


FIG. 140.—Common housefly (Musca domestica): Puparium at left; adult next, larva and enlarged parts at right. All enlarged. (From circular 71 (by L. O. Howard), Bureau of Entomology, U. S. Department of Agriculture.)

Auchmeromyia luteola.—The larva of this African fly, the "Congo floor maggot," is a blood-sucker. The larva is about two thirds of an inch long and has a dirty-white, thick, leathery, wrinkled skin, otherwise resembling that of M. domestica. The adult fly resembles the blow fly but is yellowish brown. The fly deposits her eggs by preference in dry dust in cracks in the floor of the native huts. The larvae hatch in a few days, and seek blood within a few hours, crawling out at night to feed on the sleeping natives. They are said to survive for a month without food. This is the only known instance of a blood-sucking larva which attacks man. (Other species of blood-sucking larvae infest the nests of birds and the burrows of certain mammals.)

Calliphora vomitoria (with a black bucca and reddish beard) and *C. crythrocephala* (with a brownish bucca and a black beard) are the common blow flies or blue bottle flies. They are large bloated flies, about one half inch long, with red eyes, and are bluish with a slight metallic lustre. The checks are hairy. They normally deposit ova on exposed food or decaying animal or vegetable matter of any kind, and on open wounds or ulcers of animals and occasionally of man.

Lucilia caesar, the common green bottle fly, is a smaller fly of shiny metallic green color with a bluish tinge. The cheeks are bare. Its habits are like those of Calliphona

If food contaminated with larvae of these flies is eaten, they may continue developing in the intestine and cause more or less marked disturbances (intestinal myiasis). In wounds or ulcers these flies prefer necrotic disintegrating tissue, but if this is not adequate they may invade and destroy living tissues. L. sericata (distinguished by its yellow palpi) is more actively parasitic, frequently attacking sheep on which it inflicts serious and even fatal injury. It may also attack man, and many cases have been observed in China. The larvae of both species (and also of *Phormia regina*, the wool maggot or black blow fly) have been used extensively in the treatment of osteomyelitis (Baer, 1931), although all are capable of injuring healthy tissue.

Cordylobia anthropophaga (Ochromyia anthropophaga), the Tumbu fly or African skin maggot.—This is an African fly whose larvae develop under the skin of man and animals. It is known as the Ver du Cayor. The ova are deposited in dry sand, occasionally on clothing, not directly on the skin. After three or four days the larvae merges and by means of its mouth hooks attaches itself (within 7 to 14 days) to the skin of the first animal with which it comes in contact, most often a rat or a puppy, or quite frequently a child. It then (painlessly) bores its way into the skin and produces a lesion like a boil which has a central opening through which the larvae breathes. It resembles the Ver Macaque, is rather barrel-shaped and beset with small spines. At maturity (12 to 14 days) when it is about half an inch long it leaves the body and pupates in the soil. Repeated infections result in an immunity (largely local in the skin) which prevents the development of the larva in the skin and is often associated with local anaphylactic phenomena.

Cochleomyia americana (C. hominovorax) (Chrysomyia americana) was shown by Cushing and Patton (1933) to be the imago of the parasitic "screw worm" in America. The adults resemble Lucilia but are somewhat larger and are distinguishable from them by the presence of black stripes on the thorax. The eggs, which number 250 or more, are deposited in or about the nose or ears or in open wounds of animals or man. The larvae have twelve segments and are encircled by narrow rings of minute spines which give them some resemblance to a screw. They burrow deeply into the tissues, causing extensive foul necrotic ulcerations, and may penetrate into the nasal sinuses. In a series of cases compiled by Aubertin and Buxton the mortality was 8%. The larvae mature after about 8 to 10 days, attaining a length of about 12 mm. They then leave the body and burrow into loose soil to pupate. C. macellaria, with which the preceding species has been confused, is primarily saprophagous, like Calliphora, and much less dangerous. It is distinguished chiefly by the character of the floor of the pharvnx which is ridged longitudinally, whereas in the parasitic species this is smooth. The larvae of the latter have larger spiracles and larger thicker tracheal tubes. These flies are common in the warmer parts of both North and South America. A related species with similar parasitic habits, Chrysomyia bezziana, is common in Asia and Africa, and often causes human infection in India.

Blood-sucking Muscid Flies.—Stomoxys, Haematobia and Glossina have a more or less elongated proboscis adapted for biting. Stomoxys has delicate palpi, shorter than the proboscis, and arista feathered only on the dorsal side with straight hairs. Haematobia has club-like palpi about as long as proboscis, and arista with hairs dorsally and ventrally. Glossina has thick-set but not clubbed palpi as long as the proboscis for

which they serve as a sheath. The arista is feathered on the dorsal side with branching hairs.

Stomoxys calcitrans.—The stable fly closely resembles the common housefly in size and shape. It can be easily distinguished by the black, piercing proboscis extending beyond the head. There are longitudinal stripes on the thorax and spots on the abdomen. The proboscis on examination will be seen to be bent at an angle near its base. The palps are short and slender. The wings diverge widely. The 4th longitudinal vein has a gentle forward curve.

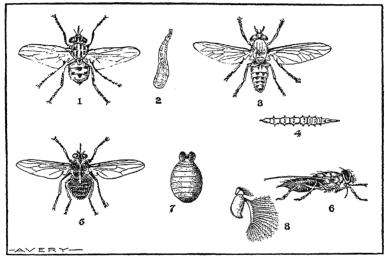


FIG. 141.—Insects in which the adult stage is important. (1) Stomoxys calcitrans; (2) S. calcitrans, larva; (3) Tabanus bovinus; (4) Tobanus larva; (5) Glossina palpalis; (6) G. palpalis, side view; (7) G. palpalis pupa; (8) Glossina palps and arista.

The female lays about 60 banana-shaped eggs in horse manure, rotting straw or other decaying vegetation. These hatch out in three days as larvae which turn into pupae in two or three weeks. After about ten days the fly emerges. The genus Stomoxys includes vicious biters. This is the fly which comes into houses before a rain, and which has given the common housefly the reputation of biting before a rain.

Stomoxys may convey trypanosome (and other) infections mechanically, like the Tabanidae. This has been proved for T. evansi and experimentally for T. brucei, T. gambiense, T. rhodesiense and several others. The old view that it transmits polio myelitis has been discredited.

Haematubia irritans, the horn fly, is a serious pest of cattle but rarely attacks man. It is about half as large as the stable fly. The palpi are much longer than in Stomoxys, being as long as the proboscis. They are thick and spatulate.

Glossina, the Tsetse Flies.—This genus is limited to tropical Africa and includes about twenty species, several of which are of great medical importance because they transmit human trypanosomiasis (sleeping sickness).

The tsetses are brownish flies a little larger than the stable fly (Stomoxys) which they resemble. The proboscis projects forward horizontally, and has a bulb at the base and a pointed tip. The palpi are long and form a sheath for the proboscis. On biting they rasp a hole through the skin with the pointed tip (labellum) and plunge the proboscis into the tissues (unlike most other biting diptera).

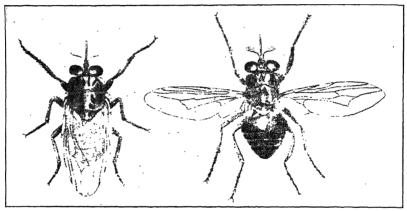


FIG. 142.—Glossina palpalis in natural resting position and with wings outstretched. (MacNeal after Doflein.)

The arista is plumose only on the upper side, and the individual hairs are themselves feathered. The wings are carried flat, closed over one another like the blades of a pair of scissors, and project beyond the abdomen. The most characteristic feature of the tsetse fly is the way the fourth longitudinal vein bends up abruptly to meet the mid-cross vein and then curves downward to run parallel with the third longitudinal vein before it turns forward again to end at the anterior border of the wing.

Glossina is peculiar also in the fact that the females are viviparous and deposit a single, very large, fully developed, yellowish brown, motile larva in shaded places in dry sandy soil. This burrows in to a depth of about two inches and immediately pupates. Moisture and sunlight are unfavorable for pupal development, especially the latter, so that pupae buried an inch deep and away from shade are killed. The period of gestation for G. palpalis is about ten days, and pupal development takes from 3 weeks (at 85°) to 12 weeks (70°). The adult flies live from 4 to 8 months. Their reproductive capacity is, therefore, very limited as compared with most diptera. They

bite during the day, even in bright sunlight. Both males and females bite and transmit the disease.

Glossina palpalis is the principal vector of *T. gambiense*, and experimentally readily transmits *T. rhodesiense* and *T. brucei*. It is a relatively large species with blackish brown abdomen and a grey thorax with indistinct brown markings. It is said to bite by preference crocodiles and other reptiles and the Situtunga antelope, but it readily bites other game and domestic animals and man. It prefers brown or black skins to white skins. For range, habits, etc. see section on Trypanosomiasis. For reasons which are not understood, in certain districts sleeping sickness does not occur although *G. palpalis* is abundant.

G. tachynoides is a smaller, darker fly showing distinct bands on the abdomen. It is found in a belt along the southern border of the Sahara from the Atlantic to Arabia. It resembles G. pal palis in its habits, and in its western range is a major vector of T. gambiense.

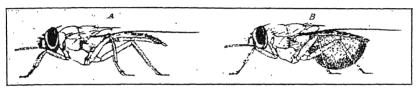


FIG. 143.—Glossina morsitans (A) before and (B) after feeding. Lateral view. (From Doflein after Austin.) (MacNeal.)

**G. morsitans** is somewhat smaller and lighter in color than *G. palpalis*. The abdomen is buff with dark cross bands which are interrupted in the midline. It bites by preference large game or domestic animals, and will bite man if these animals are not available. It has a wider range than *G. palpalis* and is not restricted to the immediate vicinity of water courses. It is the principal vector of *T. brucei* and *T. rhodesiense*.

Other proved vectors in which development of the parasite has been observed are: Of *T. gambiense*: (experimentally) *G. morsitans*, *G. pallidipes* (a natural transmitter in Uganda), *G. brevipal pis*, and *G. fusca*.

Of T. rhodesiense: G. swinnertoni (in Tanganyika) and G. brevipalpis.

Of T. brucci: (experimentally) G. brevipalpis, G. pallidipes, G. palpalis and G. tachynoides.

### Sarcophagidae

These "flesh flies" are distinguishable by the arista which is plumose to the midpoint and bare at the tip. They are usually thick-set, moderately large flies, dull colored with grey longitudinal stripes on the thorax. The abdomen is grey, checkered (in Sarcophaga) or spotted (in Wohlfartia) with black. They are viviparous. Most species deposit larvae on decaying flesh or vegetable material, but a few are parasitic in skin wounds or in the nasal or other cavities. Fatal cases have been reported. The larvae have powerful curved mouth hooks, a girdle of spines on each abdominal segment, and stigmal plates set in a deep cavity, each with three parallel vertical slits. The numerous species are difficult to identify.

Sarcophaga haemorrhoidalis is a widely distributed species which may give rise to intestinal myiasis. Cases have been reported in the United States. S. carnaria prefers to deposit larvae in the vagina when accessible.

Wohlfartia magnifica is a common cause of cutaneous myiasis in the old world. Its habits are like those of *Cochleomyia americana*. W. vigil is an American species which may be parasitic. The larvae are deposited about the eyes or on the skin, and in young children, at least, may penetrate the normal skin. Several cases have been reported in North America.

#### Oestridae

The flies of this family are called warble or bot flies. They are large flies which superficially resemble bees. The mouth parts are vestigial. They have a large head with a somewhat bloated-looking lower portion. They are often rather hairy. The larvae which develop from the eggs are parasitic in the intestinal tract, nasal cavities or subcutaneous tissues.

Dermatobia hominis (D. cyaniventris) is a large thick-set fly about 15 mm. long, with prominent head and eyes, small antennae, and a marked narrowing at the junction of the thorax and abdomen. The thorax is greyish and the abdomen metallic blue. The species is wide spread in tropical America, living in damp forested regions. When ready to oviposit the flies capture large Psorophora mosquitoes as they emerge from the pupa (and occasionally other biting diptera, or even ticks) and deposit 15 to 25 eggs on the ventral side of the abdomen, gluing them to it in such a position that the point of emergence of the larva is directed away from the mosquito. When the mosquito bites either man or other animals the warmth of the body stimulates the emergence of the larva. This burrows through the puncture wound into the subcutaneous tissue by means of its powerful mouth hooks. Here it develops into a club- or flask-shaped structure (Ver Macaque), later becoming more cylindrical (then called "torcel" in Venezuela, and "berne" in Brazil). It is girdled by several rows of spines. As the larva grows a tumor-like swelling develops with a central orifice toward which the posterior (pointed) extremity of the larva projects and through which it takes air into its spiracles. After 7 to 15 weeks the larva leaves the tissues and burrows into moist soil to pupate.

Hypoderma bovis and H. lineata, the common warble flies of cattle, and H. diana, the European deer bot fly, deposit their ova on the hairs of the legs and abdomen. The larvae, after emerging, penetrate the skin and migrate slowly through the tissues, reaching the skin of the back after about six months. Here they cause boil-like swellings ("warbles") from which the fully developed larvae finally emerge. They occasionally infect man. In Hypoderma the arista is bare, while in Dermalobia the upper border is plumose. Gastrophilus nasalis and G. haemorrhoidalis, bot flies of horses, have a similar life cycle. A number of cases of human infection have been reported. Man appears to be an unsuitable host. When the larvae reach the skin they tend to migrate aimlessly, and give rise to a "creeping eruption" similar to that caused by the larvae of the dog hook worm.

## Cutaneous Myiases

Ver Macaque.—The best known of these myiases is that due to the larva of a botfly, *Dermatobia hominis*.

The natives of most of the countries where the infection is found have called the larvae "mosquito worms" or "gusano de zancudo." The cutaneous swelling somewhat resembles a blind boil and may be as large as a pigeon's egg.

These botfly boils tend to break down and discharge a sero-purulent fluid and it is supposed that the larva, when mature, escapes as a result of the disintegration of the tumor.

In Brazil they make tobacco juice applications which cause the larva to protrude and then squeeze it out. The injection of a little chloroform into the larva with a hypodermic syringe, prior to its extraction with a forceps, makes the process less painful.

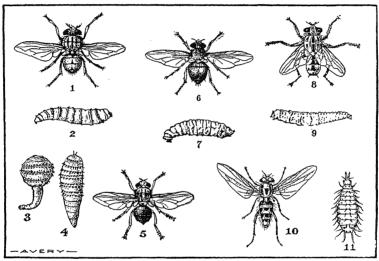


FIG. 144.—Insects in which the larval stage is important. (1) Chrysomyia macellaria; (2) C. larva; (3) Dermatobia hominis larva, early stage (ver macaque); (4) D. hominis larva, later stage (torcel or berne); (5) D. hominis; (6) Auchmeromyia luteola; (7) A. luteola, larva; (8) Sacrophaga magnifica; (9) S. magnifica larva; (10) Anthomyia pluvialis (Fannia); (11) A. pluvialis larva.

Ver du Cayor.—These maggots, which are the larvae of the *Cordylobia anthro-pophaga*, are widespread in Africa. They penetrate the skin especially of very young children who are not kept clean. The lesion rather resembles a boil.

The Screw-worm.—This is the larva of *Cochliomyia americana* (see page 552). The eggs are deposited in wounds or orifices having offensive discharges. The larvae penetrate into adjacent tissues and cause frightful destruction of all soft parts. This infection is especially common in tropical and subtropical America, and is important both in animals and man.

The larvae of many other species may secondarily infect necrotic tissue in open wounds or ulcers, and occasionally invade fresh tissue. Among the more important are Cochliomyia macellaria, Musca domestica, and species of Calliphora, Lucilia, Phormia, Sarcophaga and Fannia.

# Intestinal Myiases

In the tropics and more rarely in temperate climates, vague digestive disturbances or violent abdominal cramps with diarrhoea and vomiting may be brought about by dipterous larvae in the intestinal canal. The symptoms may be those of a dysentery and may be attended by fever and malaise.

The larvae usually obtain access to the alimentary tract on food, especially if tainted, which has been exposed to flies. It is possible for flies to deposit eggs or larvae on the skin about the anus while the individual is at stool or otherwise exposed, particularly if the skin is soiled. The larvae may then crawl into the rectum, the vagina, or even the urethra. Many species have been reported. Among the more frequent are Fannia canicularis, F. scalaris, Musca domestica, Sarcophaga, Muscina (the non-biting stable fly), Eristalis, Apiochaeta scalaris, and Piophila casei, the cheese skipper. The larvae are passed eventually in the stools or vomitus. Before making the diagnosis it is necessary to exclude the possibility that larvae which may be found in the stool have been deposited on it subsequent to its passage.

## Aural Myiases

While the larva of Cochliomyia americana, the screw worm, is that most frequently reported from the external auditory canal, many cases have been due to Sarcophaga, Calliphora, and Anthomyia (Fannia). These larvae are usually deposited in the canals of those with otorrhoea. The nasal cavities and even the eyes may be attacked. The symptoms are intense ear ache, giddiness, and possibly convulsions. The larvae tend to perforate the tympanic membrane. Instillations of to% chloroform in milk or the use of oils kills the larvae.

### DETERMINATION OF DIPTEROUS LARVAE

There are certain points in the anatomy of dipterous larvae which must be considered in determination of the genus or family of the flies concerned in the various myiases. The broad extremity is the posterior one and the tapering one the anterior. The dark hook-like processes, which may be in pairs or fused, project from the anterior or head end and above them is a pair of projecting papillae. The second segment from the head has on either side projecting hand or fan-like structures with varying numbers of terminal divisions, 4 to 40 or more. These are the anterior spiracles.

The large terminal segment has on its posterior surface two chitinized plates with 3 slits of various architecture in each. These are the posterior stigmal plates and are

the structures to which we pay particular attention in identification. In the early larval stages there is only one slit; in the second stage there are two. It is only in the fully developed larval stage that we note the characteristic 3-slit stigmal plates. The presence or absence of a rounded protuberance or button at the base of each stigmal plate should be looked for. The area carrying the stigmal plates may be sunken to form a pit.

	Key to the Larvae Causing Myiases—Third Instar $(Adapted\ from\ Banks\ after\ Fox)$
ι.	Body with spinous or fleshy processes laterally and dorsally or terminal 2
2.	Body without spinous or fleshy processes
	Body with long tail-like process.  Eristalis
	species, body about 5 mm. long
3.	Same, body about 10 mm. long
3.	pyriformOestridae 4
4.	Body truncate, broadly rounded at one end and tapering at the other (head) end. 6 Larva flask shaped, heavily spined; posterior spiracle has three distinct slits  Dermatobia
	Larva grub-like, heavily spined
5.	Three sinuous spiracular openings
_	plate
6.	But one great hook, posterior stigmal plates D-shaped with winding slits; no distinct lateral fusiform areas, tip of body with few if any conical processes Musca domestica
	One hook; stigmal plates irregularly rounded, with winding slitsStomoxys
7.	With two great hooks; slits in the stigmal plates not sinuous
	Distinct tubercles above anal area; often process around stigmal field; lateral fusiform areas usually distinct
8.	Stigmal plates on black tubercles, lateral fusiform areas distinct Ortalidae Stigmal plates barely if at all elevated; lateral fusiform area indistinct, stigmal
_	plates often contiguous or nearly so; slits long and subparallel
	Slits slender and subparallel to each other
10.	Two tubercles above anal area; stigmal field with distinct fleshy tubercles around it
	Four or more tubercles above anal area; slits of stigmal plates usually pointed at
	one end, bent, surrounded by dense chitinous ring
11.	plate
12,	Stigmal plates not in a pit
	Simuliidae (Buffalo Gnats, Black Flies).—These are small flies from 1 to 6 mm. long.

The body is stout, and the thorax is humped. The wings are broad with conspicuous

anterior veins. The legs are short, and the proboscis is short and inconspicuous. The antennae have eleven joints and are shorter than the head. Many species have been described. These flies are abundant throughout the world. The females (only) are vicious biters (by day light). They may occur in swarms so dense as to make infested districts almost uninhabitable and to cause serious destruction of live stock. The larvae, which are very characteristic (Fig. 146), require abundant oxygen. They are commonly found attached to slightly submerged rocks or vegetation in clear flowing streams; the American species noted below, in swiftly flowing mountain streams or water falls. These breeding habits explain the "patchy" distribution of the flies.

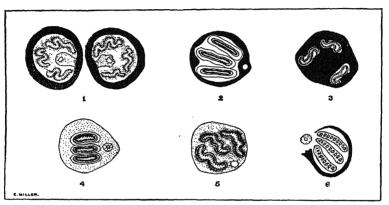


FIG. 145.—Markings of breathing slits on posterior stigmata of various dipterous larvae. I. Musca domestica, showing both sitgmata; 2. Calliphora vomitoria; 3. Stomoxys calcitrans; 4. Auchmeromyia luteola; 5. Cordylobia anthropophaga; 6. Sarcophaga magnifica.

Simulium damnosum is the intermediate host of Onchoccrca volvulus in tropical Africa. In Central America the known vectors are Eusimulium metallicum (avidum), E. ochraceum and E. callidum (mooseri). The development of the parasite is like that of W. bancrofti in the mosquito.

Chironomidae (Midges).—These are minute flies ranging from a length of 1 or 2 mm. down to almost microscopic dimensions. The wings are narrow, shorter than the abdomen, often spotted, with the first two longitudinal veins heavy, the others indistinct. They bear hairs but no scales. The antennae are relatively long and have 13 joints. The proboscis is short. Most of the species rest with the fore legs elevated. The great majority of the species are harmless. The blood-sucking species belong to the family Ceratopogoninae, and most of them to the genus *Culicoides*. These flies usually appear in dense swarms and cause troublesome irritation and itching by their bites.

Culicoides grahami and C. austeni are the intermediate hosts of the filarial worm Acanthocheilonema perstans in British Cameroons. Only the females bite, and they bite only in darkness. C. furens was shown by Buckley (1933) to be the intermediate host of Mansonella ozzardi in St. Vincent. This fly, which is a troublesome biter, is found in the coastal region from Florida to Brazil

Townsend has advanced evidence that related midges, Forcipomyia utae and F. townsendi are vectors of dermal leishmaniasis ("uta") in western Peru.

Psychodidae (Moth Midges).—These are small slender flies with very long legs. The body and wings are covered with long hairs. The wings show nine longitudinal veins which reach the margin, and cross veins only near the base. The antennae are long and hairy, and consist of 12 to 16 joints. The palpi have 4 joints. The majority of these flies have a short proboscis not adapted to biting (the Psychodinae). A relatively small group, the sand flies (Phlebotominae, genus *Phlebotomus*) are biters. They

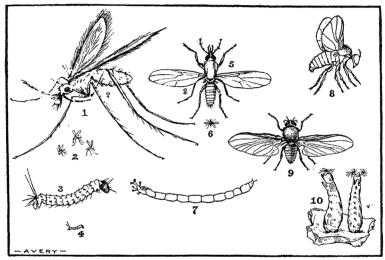


FIG. 146.—Mosquito-like insects belonging to families Chironomidae, Simuliidae and Psychodidae. (1) Phlebotomus papatasii; (2) P. papatasii (natural size); (3) P. papatasii (larva); (4) P. papatasii larva (natural size); (5) Ceratopogon pulicaris; (6) C. pulicaris (natural size); (7) Chironomus larva; (8) Attitude of a Simulium; (9) Simulium reptans; (10) Larave of Simulium.

have a long proboscis, as long as the head, and mouth parts like a mosquito. The palpi are longer than the head. The antennae have 16 constricted joints. They are further characterized by the wing venation; the second longitudinal vein bifurcates at a considerable distance from the base of the wing, instead of near the base, and has three distinct branches. When at rest the wings are raised over the abdomen at an angle of 45°. Differentiation of species is difficult and depends on slight variations in wing venation, length of the palps, etc.; thus the second segment of the palpi in P. papatasi is a little longer than the third; in P. perniciosus the segments are equal in length; while in P. minutus the second segment is only half the length of the third.

Phlebotomus papatasi is the vector of phlebotomus or pappataci fever. This species is abundant throughout the Mediterranean region, the Balkans, and Asia eastward to India. The flies hide in damp shady places during the day. The females emerge at

night to bite man or other animals. They fly only short distances and rarely rise more than a few feet above the ground. A few days after biting they deposit their ova (40 to 60) by preference in crevices of damp shaded rocks, stone fences or ruined buildings or in caves, within one or two hundred feet of their feeding places. Occasionally they may bite a second time, and deposit a second batch of ova, but their life span is short, 7 to 14 days. The life cycle occupies one to two months. The virus of pappataci fever is acquired by biting a patient during the first day of the disease. The period of incubation in the fly is about 8 to 10 days. The virus is transmitted to the succeeding generation of flies, which appear to constitute the reservoir of the infection.

P. verrucarum and P. noguchii are vectors of Bartonella bacilliformis, the cause of Oroya Fever and Verruga Peruviana in Peru (Townsend, 1914; Noguchi et al. 1929). Their distribution in the canyons of the western slope of the Andes corresponds closely to that of the disease.

Sand flies are probably the vectors of Leishmania. (See section on Leishmaniasis.) The species incriminated are: For L. donovani—P. argentipes in India, P. major var. chinensis and P. sergenti in China, and P. major, P. sergenti and possibly P. papatasi in the Mediterranean region (infantile and canine infections). For L. tropica—P. papatasi and P. sergenti in the Old World and probably P. intermedius for L. bruzitiense in South America. These species have all been infected by allowing them to bite a patient with the disease, and cyclic development of the parasite within the flies has been observed. Man and animals have been infected by injections of suspensions of infected flies and by rubbing infected flies into the abraded skin, but not by simple biting. Squashing the fly while biting seems to be necessary to convey the infection.

## CHAPTER XXIII

## THE MOSQUITOES

Mosquitoes (Culicidae) are of the greatest importance medically, not only from their influence upon health in general by reason of interference with sleep and possibly from direct transmission of disease, but, more specifically, they are the only means by which it at present appears possible to bring about infection with such diseases as yellow fever, malaria, filariasis and dengue. In addition, many diseases of animals are transmitted by mosquitoes.

The Culicidae differ from all other Diptera in having scales on their wings and generally on head, thorax, or abdomen, and the subfamily Culicinae, the true mosquitoes, differ from the other culicids by having a long proboscis.

To identify a mosquito, note the presence of a long proboscis, examine a wing and note the scales; also note the presence of two distinct fork cells and, in addition, that the costal vein passes completely around the border of the wing, making a sort of fringe with its scales. Mosquitoes undergo a complete metamorphosis, there developing from the egg a voracious, rapidly growing larva, which moults four times, transforming to pupa with the fourth moult. The pupa or nymph constitutes a non-growing or non-feeding stage in which the head and thorax are combined in an oval body, from the back of which project the siphon tubes; and tucked in ventrally is a small tail-like appendage.

The fully developed insect emerges from the pupa.

The Culicidae belong to the suborder Orthorrhapha, section Nematocera, characterized by long articulated antennae. It includes four families: Culicidae, Chironomidae, Simuliidae and Psychodidae.

## Culicidae

The culicids are divided into at least two subfamilies of which the most important is that of the Culicinae or true mosquitoes. The other subfamily is that of the Corethrinae which differ from the Culicinae in not possessing a long proboscis that is adapted for piercing.

CULICINAE. Anatomical Considerations.—Mosquitoes have three main parts of the body—the head, the thorax and the abdomen.

The head.—The space on the head behind the two compound eyes is described as consisting of two parts—that in front being called the frons, and that behind, the occiput.

The nape is back of the occiput. The bulbous prolongation of the frons which projects over the attachment of the proboscis is the clypeus. The proboscis is straight

in all mosquitoes of importance medically. In the male the puncturing parts are not sufficiently resistant to penetrate the skin. The male mosquitoes do not feed on blood but on fruits and flowers instead. The proboscis consists of a fleshy, scaled, gutter-shaped portion beneath, known as the labium, which terminates in two hinge-joint processes—the labella. At the end of the labium is a thin membrane (Dutton's membrane). It is through this that filarial embryos are supposed to pass on their way from the interior of the labium to enter the person bitten. The labium may be considered

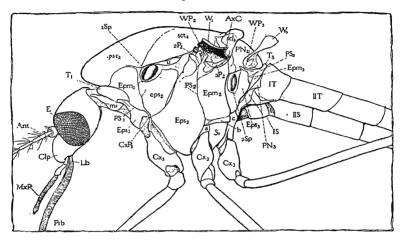


FIG. 147.—Thorax of *Psorophora*. The individual segment of the thorax to which any part belongs is indicated by the small figure placed behind and below its symbol. The abdominal segments are distinguished by Roman numerals placed before the symbols of the parts. Arabic numerals placed before symbols signify numerical order of repetition.

Ant, antenna; AxC, axillary cord of wing base; Clp, clypeus; Cx, coxa; CxP, pleural coxal process; E, compound eye; Epm, epimerum; Eps, episternum; eps, detached part of episternum of mesothorax; Eb, labium; mi, cervical sclerite; MxPlp, maxillary palpus; P, parapterum; PN, postnotum (postscutellum, metanotum); Prb, proboscis; PS, pleural suture; psc, prescutum; S, sternum; scl, scutellum; scl, scutum; Sp, spiracle; T, tergum; Ws, wing; Ws, haltere; WP, pleural wing process; a, small plate of mesopleurum bearing articulation of coxa; b, accessory plate of mesopeimerum; c, lower part of metapleurum. (After Howard, Dyar and Knab, by courtesy of Carnegie Institution.)

as the sheath of a knife, holding and protecting the slender, blade-like penetrating organs. Lying in this groove we have, from above downward, the horse-shoe-shaped labrum-epipharynx, the under surface of which is open. This when closed by the underlying hypopharynx forms a tube through which the blood is sucked up by the mosquito. In the hypopharynx, which somewhat resembles a hypodermic needle, is a channel, the veneno-salivary duct. It is down this channel that the malarial sporozoite passes. The proboscis is provided with a pair of maxillae and a pair of mandibles. These four structures with the hypopharynx and the labrum-epipharynx (six structures in all) constitute the piercing parts. There are two pairs of mandibles and two pairs of maxillae

on either side of the hypopharynx—the mandibles above and the maxillae below. The serrations of the maxillae are coarser than those of the mandibles. The sensory organs,

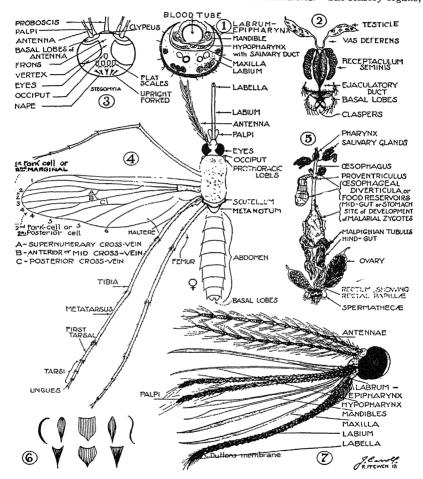


Fig. 148.—Anatomy of the mosquito. No. 6 shows various types of scales.

the palps, lie on either side of and slightly above the proboscis. These are of importance in differentiating mosquitoes and must not be confused with the antennae, which are attached above the palpi and at the sides of the clypeus. These antennae also are

of importance in distinguishing the sex of the mosquito. In the male the antennae are plumose, in the female sparsely decorated with short hairs.

The thorax.—The thorax is largely made up of the mesothorax, at the posterior margin of which is a small, sharply defined piece, the scutellum; this may be smooth or trilobed. Underneath and posterior to the scutellum is the metanotum. This term implies that the structure belongs to the metathorax whereas in reality it is mesothoracic; it is often called the postnotum or the postscutellum, the latter term being usually applied in the higher orders. The metanotum is bare in the tribes Culicini and Anophelini and has a tuft of setae in the tribe Sabethini. This holds true for the species found in the United States.

There is a pair of wings attached to the posterior part of the mesothorax and, more posteriorly still, a pair of rudimentary wings (halteres) attached to the metathorax.

The wing venation is important. The costa shows as a stout rib or vein bordering the upper side of the wing and running around the apex and lower border.

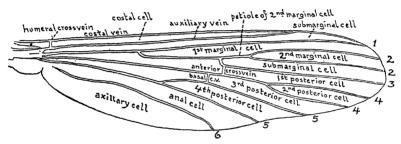


Fig. 149.—Venation of wing of Culex. (From Howard, Dyar and Knab, by courtesy of Carnegie Institution.)

Below, it has a fringe which may show spots. The location of the spots in the upper part of the costa of anophelines is of great value in differentiating species. the upper costal border the auxiliary or the subcostal vein runs to join the costa at some distance within the apex. The apex is the free end of the wing and the base that end attached to the thorax. Running parallel to the subcosta, but reaching the apex, is the 1st longitudinal vein. Below that is the 2d longitudinal vein which forks to make the 1st fork cell, also called 2d marginal cell. See Figs. 148 and 149. The third longitudinal vein originates from the second beyond the middle of the wing and is angulate at its base; the small transverse portion has been frequently called the "supernumerary cross vein." The 4th longitudinal divides to form the 2d fork cell (2d posterior cell). The 5th and 6th longitudinal veins arise from the base of the wing and run to the periphery. A small cross vein, which joins the basal part of the third vein with the fourth vein, is called the anterior or mid-cross vein. Another cross vein joins the fourth vein and the upper branch of the fifth vein and is called the posterior cross vein. The posterior or basal cross vein is usually a short distance behind the anterior cross vein; it may, however, be in line with it or even beyond it. The petiole or stalk of the second marginal cell is of importance in differentiating genera. The wings of all mosquitoes have scales. In addition, except in the genus Uranotaenia, they also possess minute hairs, microtrichia.

The three pairs of legs are attached to the thorax. Each leg has 9 parts, of which the two short ones are the basally placed coxa and the small trochanter attached to it. Then come the long femora, tibiae and metatarsi with the four segments of the tarsi terminally. The last tarsal segment ends in two claws, which in the female may be simple or uni-serrated.

The abdomen.—There are ten segments in the abdomen. The genitalia arise from the two terminal segments as bilobed processes. The posterior abdominal appendages of the female are called the cerci, those of the male the hypopygium. In the male there is a pair of hook-like appendages or claspers, between which, and ventrally situated, are the harpes, or the 10th sternites, and also a pair of chitinous processes. See Fig. 154.

Development of the Mosquito. The ova.—The egg raft of Culex, containing about 250 ova, is quite perceptible on the surface of the water as a black, scooped-out mass, about  $\frac{1}{2}$  inch in length. The eggs are set vertically in the raft. The eggs of the Acdes are laid singly.

Anopheles eggs are oval in shape with air-cell projections from either side. They are laid in triangle and ribbon patterns. The markings of these air cells vary and have been used for differentiation. The duration of the egg stage varies according to temperature and other conditions. The Anopheles are more difficult to raise than Culex or Aedes.

Larvae.—There are two great classes of larvae—the siphonate and the asiphonate. The latter are always Anopheles.

The Culex and Aedes larvae have a projecting breathing tube at the posterior extremity which is called a respiratory siphon. This projects off at an angle from the axis of the body, the true end of which terminates in four flap-like paddles. If you divide the length of the siphon by the breadth, you get what is known as the siphon index. The larva of Culex quinquefasciatus has a long and slender siphon, the larva of Aedes acgypti has a short and barrel-shaped one. When at the surface the Culex quinquefasciatus larva has its siphon almost vertical and the body at an angle of about  $45^{\circ}$ . The Aedes acgypti larva hangs more vertically. As a rule, the hairs proceeding from the sides of Culex larvae are straight and the head relatively large. There are also no palmate hairs along the sides.

The Anopheles larvae have a small head which is capable of being twisted around with lightning-like rapidity. They are darker in color and have no siphon; float parallel to the surface of the water; have long lateral branching hairs, and on the sides of each of the five or six middle abdominal segments they have a pair of palmate hairs. These palmate hairs are supposed to aid them in keeping their position on the surface of the water. The larvae are usually called "wrigglers." The duration of the larval stage is from one to two weeks according to the temperature.

Since larval characteristics serve as an important adjunct in differentiating tribes and genera and even species of mosquitoes, two larval keys are given under classification of mosquitoes. It is to be noted, however, that several of the characteristics do not appear until the last stage of larval existence, i.e., after the third moulting.

Pupae.—The pupa of the mosquito is an obtected one, there being only a closely applied chitinous coating covering it; it does not have a puparium as does the coarctate pupa of the house fly. The mosquito pupa is lighter than water while the larva is heavier.

Pupae have a bloated-looking cephalo-thorax and a shrimp-like tail—the latter being the abdomen.

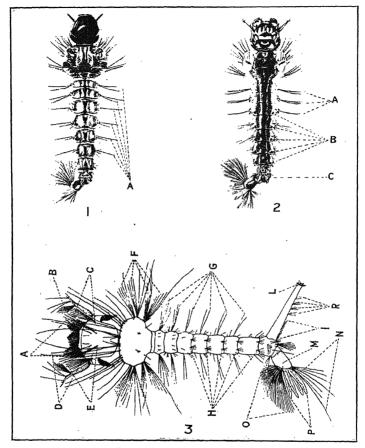


Fig. 150.—Mosquito larvae.

I. Anopheles barberi. (A) The plumose lateral hairs on the first six segments of abdomen. Note small single hairs on head.

2. Anopheles punctipennis. (A) The plumose lateral hairs on the first three segments of abdomen; (B) the five pairs of dorsal palmate tufts; (C) sessile air-tube.

3. Diagram of culicid larva. (A) Mouth brushes; (B) tuft of antenna; (C) ante-antennal tuft; (D) lower frontal tuft; (E) upper frontal tuft; (F) thoracic hair tufts; (G) abdominal lateral tufts; (H) abdominal subdorsal tufts; (I) pecten of air-tube; (R) ventral hair tufts of air-tube; (L) apical spine of air-tube; (M) anal segment; (N) subdorsal tufts of anal segment; (O) ventral brush; (P) anal gills. (After Howard, Dyar and Knab, by courtesy of Carnegie Institution.)

The duration of pupal life is short—only one to three days. At the end of this time the pupa comes to the surface and straightens out. The integument then splits dorsally and the perfect insect emerges. It dries its wings for a time on its raft-like pupal skin and then flies away.

Thus the metamorphosis of the Anopheles mosquito under favorable weather conditions takes from two to three weeks; one to three days for the egg stage, ten to fourteen days for the larval stage, and two to three days for the pupal stage.

Dissection of the Mosquito.-The easiest way to secure a mosquito for dissection is to use an ordinary test tube. Slipping the open end of the test tube over the resting mosquito, by a slight movement, the insect will fly toward the bottom. Then quickly insert a cotton plug. If it is not desired to study the scales, the best way to kill the mosquito is by striking the tube sharply against the thigh; but if it is also desired to study the scale characteristics it is better to put a drop or so of chloroform on the lower part of the cotton plug. The vapor falls to the bottom of the tube and kills the mosquito. Take the mosquito out. pull off legs and wings, and then place the body in a drop of salt solution on a slide. It has been recommended to smear the surface of the slide with bile, wiping off the excess, before commencing the dissection in the salt solution. Then hold the anterior end of the thorax by pressure of a needle. With a second needle in the other hand, gently crush the chitinous connection between the sixth and seventh segments of the abdomen. Then holding the thorax in place, steadily and gently pull away the last segments. If this is done properly, a Stitt.) (From P. H. Reports.) delicate gelatinous white mass will slowly

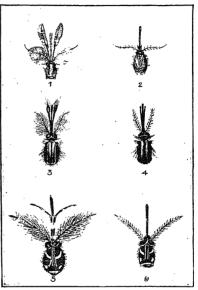


Fig. 151.—Heads of mosquitoes: 1 and 2, male and female Culex quinquefasciatus; 3 and 4, male and female Anopheles; 5 and 6, male and female Aedes aegypti. (After

float out in the salt solution. One should be able to secure the alimentary canal as far up as the proventriculus, which is just anterior to the stomach. The malarial zygotes develop in the stomach. Proceeding from before backward, we have the proventriculus, which is a sort of muscular ring at the opening of the stomach or mid-gut and marks the separation of the stomach from the oesophagus. Opening into the lower part of the oesophagus are the oesophageal diverticula or crops, which are food reservoirs. Occasionally in a dissection we pull out these structures which are three in number.

Leading from the stomach we have a hind-gut, which ends in the rectum.

This has a posterior dilatation or rectal pouch which usually has three or four rather marked anal papillae.

Taking origin at the posterior end of the stomach and festooning the hind-gut are five longitudinal tubes—the Malpighian tubules. These are characterized by large granular cells with a prominent refractile nucleus. They are regarded as the renal

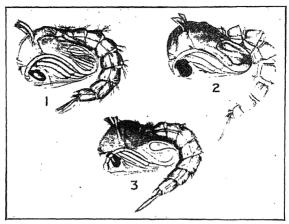


Fig. 152.—Mosquito pupae. (1) Culex pipiens; (2) Aedes aegypti; (3) Anopheles punctipennis. (After Howard, Dyar, Knab, by courtesy of Carnegie Institution.)

structures. It is in these tubules that the embryo of the Filaria immilis of the dog develops. In the female mosquito, the parts withdrawn may seem to be largely made up of the white oval ovaries. These are connected with the spermathecae, in which the spermatozoa are stored after fecundation by the male. In the male the testicles are

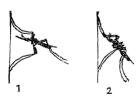


Fig. 153.—Resting posture of mosquitoes: 1 and 2, Anopheles; 3. Culex pipiens. (After Sambon.) (From P. H. Reports.)

quite distinct. Next to the examination of the stomach for zygotes, which appear is wart-like excrescences on its outer surface, the most important structures are the salivary glands, where the malarial sporozoites are found. The easiest way to dissect out the salivary glands is to press down firmly, but gently, on the anterior part of the thorax, and then with the shaft of a second needle, pressing on the head, to gently draw the head away from the thorax, so that by this expression and traction

movement you extract them with the head segment. They are very minute and are to be recognized by their exceedingly highly refractile appearance. To examine for sporozoites cover the glands protruding from the neck with a cover glass and search with one-sixth objective for narrow, curved bodies in the substance of the glands. If they are present try to smear out the glands between the cover glass and slide by pushing the cover glass along; then, withdrawing the cover glass, dry quickly and stain the smear on slide or cover glass with Wright's stain.

The sporozoites are narrow falciform bodies about  $12\mu$  in length, with a central chromatin dot.

A matter about which there is dispute is as to whether the salivary glands communicate with the alimentary canal. Theobald states that there is no connection between them.

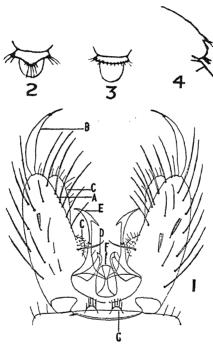


Fig. 154.—(1) Diagram of the male genitalia of Aedes. (A) side piece; (B) clasper; (C) lobes of side piece; (D) 10th sternite; (E) claspette; (F) mesosome; (G) 9th tergite. (After Howard, Dyur and Knab, by courtesy of Carnegie Institution.) (2) Culex sp. The trilobed scutellum and the metanotum. (3) Anopheles sp. The rounded scutellum and the metanotum. (4) Sabethine species. Side view of the metanotum showing the tuft of setae.

Epidemiological Importance of Species Determination.—In considering whether danger might arise from the introduction into a region of a case of yellow fever, malaria or filariasis, it is essential to ascertain if genera and species capable of transmitting these diseases are present. Very definite information as to the identity of mosquitoes can be obtained, if mosquito ova are available for study, by examining specimens in the several stages of development from ovum to imago. All points concerned in species

differentiation are thus made available. Having determined the species from the characteristics of egg, larva and pupa, examination of the imago becomes a process of verification

Classification of Mosquitoes.—The classification of mosquitoes is steadily undergoing changes following progress in the science of entomology, and the discovery of new species. Theobald's classification that has been the accepted one for many years is now found wanting and even misleading. The modern development in the study of larvae

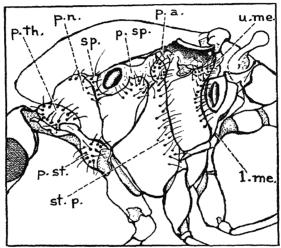


Fig. 155.—Schematic diagram of the thorax of a mosquito to show areas in which bristles may be present.

l.me., lower mesepimeral bristles; p.a., prealar bristles; p.n., pronotal bristles; p.sp., post-spiracular bristles; p.st., prosternal bristles; p.th., prothoracic bristles; sp., spiracular bristles; st.p., sternopleural bristles; u.me., upper mesepimeral bristles.

and their characteristics has helped much to bring order out of the chaos that existed. The scope of this book does not permit us to include the large keys necessary to identify the mosquito species of the world. In order, however, to give the student some idea of how this identification is made, we have selected two keys for the adult mosquitoes dealing with the genera found in the United States and with species of the genus Anopheles found in this country; also two keys of the same scope dealing with larval characteristics.

In the larger keys a point of major importance in the identification of genera is the distribution of bristles on the thorax. This is rather difficult to make out. However,

in the following simplified key some of these characteristics have been included in parentheses.

The generic key for adults holds true only for the United States as there are some Old World Sabethines that do not conform with the classification. Even in tropical America there are Sabethines with a nude metanotum.

Key to the Tribes and Genera of the Mosquitoes of the United States. Adults.  $(After\ Dyar)$ 

	. 5 . 5 . 7
I.	Metanotum with a tuft of setae
	Metanotum nude
	Wings with the second marginal cell less than half as long as its petiole
3.	Proboscis rigid, down curved. (Pronotal bristles absent. Row of stout spiracular bristles. Scutellum not trilobed.)
	Tribe Megarhinini Genus Megarhinus Robineau-Desvoidy
	Proboscis flexible, straight. (Only a single pronotal and one spiracular bristle.) Wings without microtrichia. Tribe Uranotaenini
	Genus Uranotagnia Lynch Arribalzaga
4.	Scutellum rounded, not lobed. Palpi of both sexes as long as the proboscis Tribe Anophelini
	Genus Anopheles Meigen
	Scutellum distinctly trilobed
5.	Lower side of base of first vein pilose. (Spiracular bristles present. Postspiracular
·	bristles absent.)
	Without this character
6.	Fourth joint of fore tarsus very short. (Spiracular and postspiracular bristles
••	absent. Pronotal bristles two stout setae.)Genus Orthopodomyia Theobald
	Fourth joint of tarsus longer, normal
7-	Second joint of antennae very long in both sexes; antennae of male similar to those
	of female. (Spiracular, postspiracular and lower mesepimeral bristles absent.
	Pronotal bristles more than two.)
0	Second joint of antennae short; antennae of male dissimilar to those of female 8
٥.	Abdomen of female blunt, with short cerci; hypopygium of male with the side pieces
	curved down, or shortly projecting. (Spiracular and postspiracular bristles absent. Pronotal bristles more than two.)
	Abdomen of female pointed, cerci exserted; hypopygium of male with the side pieces prominently projecting, straight. (Postspiracular bristles present.)
g.	Wing scales narrow, normal. (Lower mesepimeral bristles one, rarely two.) Genus Culex, Linnaeus
	Wing scales distinctly large and broad (Lower mesepimeral bristles three or four) Genus $Mansonia$ Blanchard
ro.	Abdomen of female with the eighth segment wholly retractile, nude; male claspette
	with multiple terminal appendages. (Spiracular bristles present.)
	Genus Psorophora Robineau-Desvoidy
	Abdomen of female with the eighth segment only partly retractile; male claspette
	with only a single appendage, or claspette wanting. (Spiracular bristles absent
	Pronotal bristles more than two.)

For the systematic study of mosquitoes of Central America, the West Indies and North America, the reader is referred to the Monograph by Howard, Dyar and Knab; and for the study of the old world species to the various publications of F. W. Edwards, of England, Theobald and others.

	LARVAE. (After Howard, Dyar, and Knab)
ı.	Anal segment without ventral brush, the hair tufts all paired Tribe Sabethini Genus Wyeomyia
2.	Anal segment with unpaired ventral median brush
3∙	Air-tube distinctly elongate
	Head nearly circular or transverse 4
4.	Mouth-brushes of lamellate prehensile plates
	Mouth-brushes normal
5-	Air-tube without pecten 6
	Air-tube with a well developed pecten
6.	Air-tube with the outer half attenuated
	Air-tube cylindrical or fusiform; antennae small, slender Genus Orthopodomyia
7-	Air-tube with but a single pair of ventral (posterior) tufts
	Air-tube with several pairs of ventral tufts, mouth-brushes normal with no pre-
	hensile hooked lamellae
8.	Air-tube pecten produced into long hairs; hair tuft close to baseGenus Culiseta
	Air-tube pecten of short scales or if produced the hair tuft remote from base 9
9.	Mandible angularly projecting laterally
	Anal segment ringed by dorsal plate, with ventral hair tufts piercing the ring.
10.	Genus Psorophora
	Anal segment not ringed, or if so with the hair tufts posterior to the ring

The only genera of importance from a medical point of view are Anopheles, Aedes, Culex and Mansonia.

Genus Aedes

Anopheles.—Determination of species.—In addition to the generic characteristics given in the key there are several others that hold true more or less exclusively for this genus. The anopheline larvae are asiphonate surface feeders and lie parallel to the surface of the water. As a rule the pupa of Anopheles rests with the long axis of the first two abdominal segments nearly parallel to the surface of the water, while in Culex and Aedes it is usually more nearly vertical. The eggs are provided with floats. The imago as a rule has spotted wings. In the female the palpi are as long as the proboscis which is always straight in this genus. The body of Anopheles when resting on a wall forms a straight line at an angle of about 45°. It resembles a bradawl. Most species are twilight feeders. Many species hibernate as adults, and there is considerable evidence that P. vivax may survive the winter (at least in milder temperate climates) in hibernating A. maculi pennis.

Transmitters of Malaria.—The species listed are all proven vectors of malaria except barberi and walkeri. However, the fact that the malarial parasite can develop in a given species of mosquito and that the latter can infect man under experimental conditions

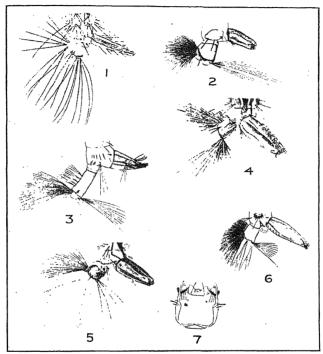


Fig. 156.—Larval characteristics demonstrating points in the keys. The posterior abdominal segments. (1) Wyoomyia smithii. Note absence of ventral brush from anal segment. (2) Megarhinus portoricensis. Note absence of pecten. (3) Mansonia titillans. Note attenuated outer half of air-tube, also absence of pecten. (4) Culiseta inornatus. Note air-tube pecten produced into long hairs; hair tuft close to base. (5) Aedes acgypti. Note: The ventral hair tufts on anal segment do not pierce the partial plate. (6) Psorophora floridense. Note anal segment ringed by plate, with ventral hair tufts piercing the ring. (7) Head of larva. Megarhinus portoricensis. Note the mouth-brushes consisting of lamellate prehensile plates. (After Howard, Dyar and Knab, by courtesy of Carnegie Institution.)

does not prove that the species is of practical importance as a transmitter under natural conditions. The species must be present in abundance, but its importance depends upon many other factors also, such as the habits of the mosquitoes, their preference for human or for animal blood, their tendency to enter houses, the time at which they bite, the distance of their breeding places from human habitations and the usual length of their

flight, and the ability of the individual insect under the weather conditions in a given locality to survive the incubation period of the parasite.

# KEY TO THE UNITED STATES SPECIES OF ANOPHELES ADULTS. (After Dyar)

ADULTS. (After Dyar)	
1. Tarsi marked with white	
Tarsi without white markings	3
2. Last three hind tarsal segments all white	4
Fifth hind tarsal segment has a black ring	5
3. Wings with white spots	
Wings with black spots or none	7
4. Second hind tarsal segment largely black; white rings on mid-tarsi	
albitarsis Lynch Arribalzaga*	K
Second hind tarsal segment largely white; mid-tarsi without white rings	
argyritarsis Robineau-Desvoidy	ļŧ
Second hind tarsal segment largely white; white rings on mid-tarsidarlingi*	s
5. Second hind tarsal segment one half black; penultimate segment of female palpus	
largely black Wiedemann	
Second hind tarsal segment one fourth (or less) black; penultimate segment of	
female palpus largely white	ŧ
6. Wings with a white spot at outer third of costa 8	
Wings without this spot	
7. Wings with black spots, usually distinct	
Wings with indistinct black spots or none, usually absent	)
8. Palpi marked with white, third vein extensively white in the middle	
pseudo puncti pennis Theobald	
Palpi wholly black; third vein wholly blackpunctipennis Say	
9. Wings with a coppery spot at apex on fringe	
Wing-fringe black at apex as elsewherequadrimaculatus Say	
10. Mesonotum rounded, but little elongatebarberi Coquillett	
Mesonotum distinctly elongate	
11. Palpi of female with whitish rings at bases of jointswalkeri Theobald	
Without distinct white rings on palpi; body blackish; hairs of mesonotum dark-	
brown	
* Important transmitters of malaria in tropical America not found in the United	
States.	

Thus A. punctipenmis and A. crucians, abundant within their range, prefer animal blood (are "zoöphilous") and are of minor importance as transmitters of malaria compared with A. quadrimaculatus which bites man and animals indifferently. Different races of the same species of mosquito may vary in their habits. In the case of A. maculipennis, for example, in certain localities in Europe the mosquito is zoöphilous and malaria is uncommon, while in other (sometimes neighboring) regions "androphilous" strains occur and malaria is abundant, especially if the insects pass the winter within dwellings instead of hibernating. As a rule the androphilous races have eggs with barred markings on the upper surfaces, while the zoöphilous races have uniformly colored eggs. In regions in which there are few domestic animals, however, the zoöphilous races are driven to bite man and become important vectors, as in certain regions in the Danube

valley and in central Russia. As living conditions improve in such regions and animal husbandry is more extensively practiced, these races revert to their natural hosts and malaria largely disappears.

#### LARVAE. (After R. C. Shannon)

- 2. (a) Abdomen with seven pairs of dorsal palmate tufts, the pair on first segment small......albimanus
  - (b) Abdomen with six pairs of dorsal palmate tufts. \quad \begin{align\*} quadrimaculatus \quad walkeri \quad atropos \\ (c) \text{ Abdomen with five pairs of dorsal palmate tufts} \quad \qquad \quad \quad \quad \quad \quad \qq \quad \

- maculipennis

Lateral plate with 17-22 (usually 6-7µ long) teeth.....punctipennis

The type of breeding place of the particular species is also of fundamental importance. Thus A. quadrimaculatus in the United States breeds in quiet pools or puddles. Thorough drainage of such areas largely eliminates this mosquito and with it malaria. The reverse is true of some other species in other localities. Thus A. maculatus breeds in open flowing streams, and in certain districts in Malaya it has been possible by damming up the streams and producing pools to eliminate this vector and replace it with harmless species. In Europe many of the races of A. maculipennis which transmit malaria breed indifferently in flowing or stagnant water, and drainage projects which are successful in America have proved quite ineffective. The breeding places of the same species may differ in different localities. Thus A. ludlowi breeds in salt water in Java, and it is limited to the coast, but in Sumatra it breeds in fresh water and is an important transmitter through much of the island.

Although it has been shown that some *Anopheles* mosquitoes are capable of traveling considerable distances (even 20 miles), as a rule their range of flight is small (under a mile), and they are rarely found in large numbers unless there are suitable breeding places in the immediate vicinity. High humidity also favors human infection because in a period of hot dry weather most of the adult mosquitoes perish before they become infective.

For reasons which are not well understood the same species may be an important transmitter in one region (e.g., A. subpictus (rossi) in the East Indies) and practically harmless in another (India) even though it is abundant. Even in the same locality a change in local conditions which favors the propagation of the mosquito may result in converting a species previously harmless into a dangerous transmitter. This occurred when the cultivation of rice was introduced into Sumatra after the war. An epidemic of malaria promptly broke out, transmitted by A. hyrcanus. It had been known that

this species was present, but it had been considered harmless because it breeds abundantly in the rice fields of Java and other neighboring islands and there it is not a transmitter. Such points can be determined only by local epidemiological studies and not by laboratory experiments or by analogy from conditions in other regions.

Anopheles albimanus.—A medium-sized black .Inopheles, the tip of hind tarsi white with a black spot on last joint. Legs blackish, the fore tarsi with white rings at apices of the first three joints; hind legs with apex of second, and the third to fifth joints white, a black mark on the fifth joint. More than one half of second segment black. Wings with black and yellowish scales, two large yellowish spots outwardly on costa; other veins with small dark spots alternating with pale scales. Palpi long, dark, last joint and base of penultimate one white. This is the principal vector of malaria in tropical America.

Anopheles punctipennis.—Tips of femora and tibiae with small white spots. Wings with scales black except in certain spots as follows: A large one at outer third of costa and a smaller one at apex, both involving second vein; one on third vein in the cell, on the stem and middle of both forks; at base and middle of fifth vein. Widely distributed throughout North America, it prefers to bite large mammals rather than man, and is regarded as a relatively unimportant malarial vector.

Anopheles quadrimaculatus.—A medium-sized blackish Anopheles with black-spotted wings. Tips of femora and tibiae whitish. Wings with the scales black, forming four dark spots by being thickly placed as follows: Base of second vein in the cell; on the cross vein and forks of second and fourth veins. Apex of wing uniformly dark. The principal vector in the United States. It occurs in the eastern states and the Mississippi Valley from the Gulf northward to New Hampshire and Wisconsin.

Anopheles maculipennis.—A medium-sized blackish Anopheles with black-spotted wings. Tips of femora and tibiae whitish. Wings with the scales black, forming four dark spots by being thickly placed as follows: Base of second vein in the cell; on the cross vein and forks of second and fourth veins. Tip of the wing with a brassy reflection in the fringe. It is widely distributed in Europe, northern Africa, western and central Asia, Alaska, Canada and western United States. It is important as a transmitter wherever malaria occurs within its range.

Anopheles crucians.—A medium-sized blackish Anopheles with mottled wings. Legs black with pale knee spots. Wings with a small yellowish-white spot at apex and fringe; other scales mostly black, forming spots at the bases of the forked cells and three on the sixth vein, separated by pale scales. The palpi of the female have the last joint whitish and a ring at base of penultimate joint. A relatively unimportant vector, occurring in the South Atlantic and Gulf states and the Mississippi Valley.

Anopheles pseudopunctipennis.—A medium-sized blackish Anopheles with white-spotted wings. Legs black, knee spots yellowish-white. Wings spotted black and white; costa black with three white patches; third vein broadly white in the middle. Palpi of female with white rings at the bases of the joints. It greatly resembles punctipennis, but is not really closely allied thereto. It is found in the southwestern United States, Central America and western South America to Argentina, where it is an important transmitter.

Another important transmitter of malaria of the New World is:

A. argyritarsis.—It is a South American species. Black costa with two distinct and several smaller white spots. Dark-brown palps with two narrow bands and a white tip. Legs with last three hind tarsal segments white.

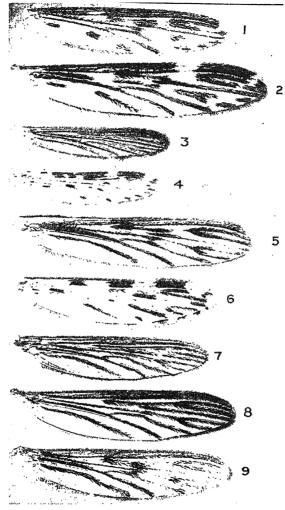


Fig. 157.—Wings of Anopheles mosquitoes shown to the same scale. (1) A. crucions; (2) A. punctipennis; (3) A. barberi; (4) A. albimanus; (5) A. maculipennis; (6) A. pseudopunctipennis; (7) A. atropos; (8) A. walkeri; (9) A. quadrimaculatus. (After Howard, Dyar and Knab, by courtesy of Carnegie Institution.)

The following Old World species are also important transmitters of malaria:

A. funestus.—Wings with four yellow spots on a black costa and two black line spots on third longitudinal vein. Palps with three white rings. Proboscis unbanded. Legs with faint apical bands. Common in tropical Africa and India.

A. gambiae (A. costalis).—Costa black with five or six small yellow spots. Palps with two narrow white bands and white tip. Femora and tibiae with yellowish spots. Apical tarsal bands. Common in tropical Africa and Arabia, it has been introduced recently into Brazil. Both species are dangerous transmitters of malaria (and potential vectors of filaria). In one district Ross found 24% of A. gambiae infected.

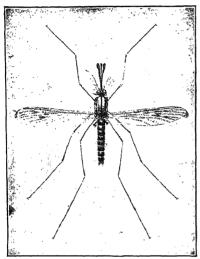


Fig. 158.—Anopheles maculipennis, male. (After Castellani and Chalmers.) (From P. H. Reports.)

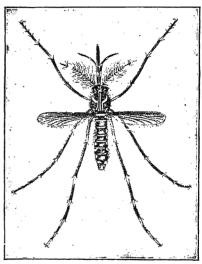


Fig. 159.—Aedes aegypti, male (Stegomyia fasciata). (From P. H. Reports.)

- A. stephensi.—Costa with four broad black spots separated by narrower yellowish spots. The corresponding second spot on the second long vein is narrower and divided into two unequal parts by a light spot (the smaller distal). Yellowish white scales over the dorsal surface of the abdomen, thorax and head. Legs black, the femora and tibiae spotted with white. White bands at intertarsal joints. Last hind tarsal segment black. It is abundant in India and a dangerous transmitter.
- A. maculatus.—Somewhat similar to the preceding. The abdomen is dark brown with golden brown hairs over the dorsum, and yellowish white scales over dorsum of thorax and head. The second spot on first long vein is divided into three parts by two pale spots. The white bands between the tarsal segments are broader and the last hind tarsal segment is white. Common and a dangerous transmitter in India, southeastern Asia and the East Indies.

**Aedes** and **Culex** are of great interest medically because species of these genera transmit filariasis, dengue and yellow fever. *Culex* mosquitoes are the principal vectors of bird malaria.

In contrast to the Anopheles the ordinary Cules has the following characteristics: The larvae have a siphon and the body rests at an angle of about 45° with the surface of the water. The wings of the imago are as a rule not spotted. In the female the palpi are shorter than the proboscis. In the resting position Cules allows the abdomen to droop, so that it is parallel to the wall. The angle formed by the abdomen with head and proboscis gives a hunchback appearance.

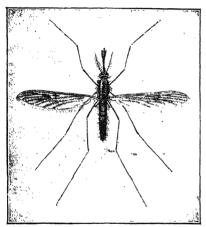


FIG. 160.—Anopheles maculipennis, female. (Castellani and Chalmers, after Austen.) (From P. H. Reports.)

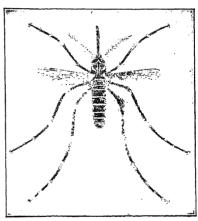


Fig. 161.—Aedes aegypti, female (Stegomyia fasciata). (From P. H. Reports.)

Culex quinquefasciatus (C. fatigans) is the most important vector of filariasis (W. bancrofti). It is a medium-sized reddish brown mosquito. Legs and proboscis have blackish scales; the femora are pale brown at base. Abdomen blackish above, with moderate basal segmental whitish bands, separated from the lateral spots. Wing scales are narrow and hair-like, wholly dark.

The larvae occur in artificial receptacles most frequently, but are also found in ground pools, even far from habitations.

This is the common house mosquito of the tropics, extending in the Americas to about latitude 35° north and south. It is active at night.

Culex pipiens, very similar to the preceding species, is the common house mosquito in temperate regions. It is a vector of filariasis in Egypt, China and Japan.

Aedes aegypti (Stegomyia fasciata, S. calopus) is the principal vector of yellow fever and dengue, and is therefore of extreme medical importance.

It is small in size, blackish-brown in color with silver stripes on thorax, abdomen and legs. The dorsal surface of the thorax is marked with two parallel lines with curved silver-white lines outside (lyre marking). The proboscis is black, the tips of the palpi white. There are silvery scales on clypeus.

These mosquitoes are often called domesticated, since they are observed to breed and pass their lives in the immediate environment of man and further to be distinctly urban, rather than rural, in their distribution. For their breeding places they choose artificial collections of water, such as cisterns, barrels, pails, bottles, cans or empty tire casings, in or near dwellings.

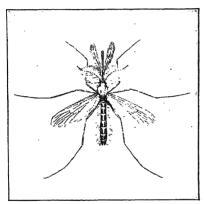


FIG. 162.—Culex quinquefasciatus, male. (After Howard.) (From P. H. Reports.)

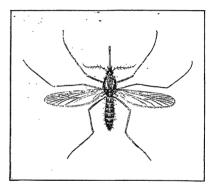


Fig. 163.—Culex quinquefasciatus, female. (After Howard.) (From P. II. Reports.)

A. aegypti is a vicious feeder and very alert. Only the female bites, blood apparently being necessary for ovulation. It feeds especially during the morning and afternoon hours—much less commonly at night unless there is a light. To become infected with yellow fever virus it must take blood from a yellow fever patient in the first two or three days of the disease. After sucking the blood of a yellow fever patient the mosquitoes cannot transmit the disease by biting a person non-immune to yellow fever for a period of twelve days. After this time the mosquito remains infective for its life—in one instance fifty-seven days.

Aedes albopictus (Stegomyia scutellaris) has a single silvery stripe down the center of the thorax. It breeds particularly in receptacles about the house. It is common in the orient. It has been proven to be a vector of dengue and (experimentally) of yellow fever.

Recent work has shown that several other species of Aedes and related genera may convey yellow fever experimentally and probably under natural conditions. Among

these (Rindle, 1933) are (in Africa) Acdes luteveephalus, A. stokesi, A. vittatus, A. simpsoni, Mansonia africana, Eretmopodites chrysogaster, and Culex thalassius; and in Brazil, A. scapularis, A. tacniorhynchus, and A. fluviatalis, which is believed to have been the vector in one epidemic.

Aedes variegatus (Stegomyia pseudoscutellaris) resembles A. albopictus but has white bands only at the sides of the abdominal segments. It is widely distributed in the Pacific Islands, in which it is the vector of filariasis (non-periodic strain of W. bancrofti). It bites by day.

A. togoi and A. chemulpoensis of Japan are effective filarial vectors, but in A. aegypti and A. albopictus development of the parasite is incomplete.

**Psorophora.**—Species of this genus have been incriminated as being passive carriers of larvae of a botfly (*Dermatobia hominis*). These larvae when the mosquito alights on the skin of man emerge from the egg case, penetrate the skin and set up a cutaneous myiasis. See page 556.

**Uranotaenia.**—A small genus, mainly of tropical distribution. The larvae live in ground pools and have a superficial resemblance to *Anopheles*, from the elongated black head and the habit of lying flat in the water, although the larvae are not surface feeders. The adults are ornamented with lines of metallic blue scales.

Megarhinus.—A genus of large showy insects of tropical and subtropical distribution. The adults do not bite, the proboscis being curved and adapted to extract honey from flowers. The larvae found in tree holes and similar locations feed entirely on other mosquito larvae. The species of this genus should be classed as strictly beneficial to man. On account of their restricted habitat the species are rare.

Mosquito Eradication.—Mosquito eradication being in practice a problem of engineering and municipal administration rather than of medicine, it will suffice here to indicate the means employed, according to circumstances, in ridding a district of mosquitoes.

In rural districts, where the malaria-carrying mosquito breeds, the measures applicable are: (1) Removal of collections of stagnant water suitable for breeding by surface or subsoil drainage, by permitting free access of the tide water, or by filling in as in the case of small ponds and wells; (2) introduction of fish which prey on larvae and pupae; (3) clearing away aquatic vegetation from the banks of streams and ponds, and (4) use of physical or chemical larvicides, as oil or Paris green.

In urban and suburban districts, where the mosquitoes transmitting yellow fever, dengue and filariasis are likely to breed, the measures are:  $(\tau)$  Piping the water supply to remove need of cisterns; (2) screening, covering and oiling all water containers; (3) removal of all rubbish that may hold water, as bottles and tin cans; (4) drainage of surface collections of water, or, where that is not possible, (5) employment of larvicidal measures; (6) as a substitute for these measures it has been shown that the introduction of the small mosquito-destroying fish into cisterns, tanks, etc., will prevent the breeding of mosquitoes. Often fish cannot be depended upon to destroy all larvae in natural bodies of water where larvae and pupae are protected by surface vegetation.

A number of chemical agents may be employed as larvicides (See page 890). The so-called Panama larvicide formulated and used successfully by Mason is compounded as follows: Add 200 pounds powdered resin to 150 gallons crude carbolic acid. Heat mixture to 212°F. until uniform liquid is obtained. Dissolve 30 pounds caustic soda

in 6 gallons of water and add to mixture, stirring briskly. Keep at boiling point until a sample immediately emulsifies with water. This larvicide in a r to 1000 emulsion kills mosquito larvae in one to five minutes; in a r to 5000 emulsion in 30 minutes. It should not be used with oil or to dilute oil as the soapy characteristics interfere with satisfactory filming. Efficacy is impaired by exposure to air. Costs 25 to 30 cents per gallon. It is ordinarily sprayed or sprinkled in 10% emulsion to form not less than 1 to 5000 emulsion with the water treated.

## CHAPTER XXIV

## POISONOUS SNAKES AND LIZARDS

SNAKES belong to the Class Reptilia and the order Squamata, suborder Ophidia. The two families to which poisonous snakes belong are the Colubridae (colubrine snakes) and Viperidae (viperine snakes).

Although the toxicity of the venom and the amount normally present are matters of great importance in estimating the lethal powers of species of poisonous snakes, the principal feature to be considered is the ability of the fangs to introduce venom into the tissues of the animal bitten. For example, in the Opisthoglypha there are fangs attached to the maxilla but these are placed posteriorly to the solid teeth in front, so that, since the venom cannot be inoculated, these snakes are from a practical point of view non-poisonous. Then, too, snakes in which the fangs are so situated have only a small poison gland and their venom is of low toxicity. In dangerous snakes the poison fangs are placed anteriorly, attached to the maxilla, which, in the poisonous Colubridae, is long and lies horizontal and, in the Viperidae, is short and lies vertical.

The non-venomous snakes are in the Aglypha series and have solid teeth. There has been a question as to a toxic saliva of some aglyphs but this is probably an allergic manifestation. With the Protereoglypha, where belong the dangerous snakes, we have grooved or canalized poison fangs, attached anteriorly to the maxilla.

Colubridae.—The three series noted above belong to this very large family. The poisonous species belong either to the Hydrophinae (sea snakes), which have an eelshaped tail and a rather flattened body, or to the Elapinae (land snakes), which have a round tail. As a rule, sea snakes live in salt water near the shore, but such snakes have been reported from fresh water lakes in the Philippines. They are of importance in the tropics and are a source of danger to fishermen. While their venom is extremely toxic, and their fangs situated anteriorly, the danger from them is minimized by their small heads and relatively inefficient bite. The Elapinae have short, strong fangs anteriorly located, and behind them small grooved (not canalized) teeth. The poison gland, which is the homologue of the parotid, has a duct located in the upper lip and terminating in a papilla. The poison duct does not enter the fang lumen but empties into muscular folds which surround the base of the fang, hence breaking off of a fang does not necessarily injure the duct. There is a succession of teeth in snakes, so that a new fang grows out if the original one is extracted.

Many of our harmless snakes such as the garter-snake and blacksnake belong to the Colubridae.

The cobras belong to the subfamily Elapinae and are best known by a neck-like expansion or hood. The only poisonous colubrine snakes in the United States are the beadsnake (*Elaps fulvius*) often called the Florida coral snake, and the Sonoran coral (*Elaps euryxanthus*).

The beadsnake is black with about seventeen broad crimson bands bordered with yellow. Although small, they are very venomous. The upper jaw has anteriorly grooved fangs, which appendages are not present in the non-poisonous coral snakes, these latter having teeth in the upper jaw so that the wound shows four rows of punctures instead of two rows and one larger puncture on each side to mark the entrance of the fangs.

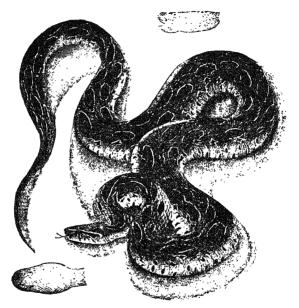


Fig. 164.—Daboia russelli. (After Mense.)

In Asia there are many important poisonous colubrine snakes, the cobra (Naja tripudians), the King cobra (Naja bungarus) and the kraits (Bungarus fasciatus).

All of the Australian poisonous snakes are colubrines.

#### SNAKES OF THE UNITED STATES (STILES)

- (B) Pupil of eye circular; pit absent; double row of ventral scales posterior to the vent.
  - (a) Color: yellow, black, yellow, red, in bands............ Coral snake (poisonous).
  - (b) Color: black, yellow, black, red, in bands......False corals (not poisonous).

Viperidae.—The viperine snakes are characterized by a broad head, narrow neck, short and stumpy tail and a short upper jaw which, with the fangs, is directed obliquely backward. The rattlesnake (Crotalus), the copperhead (Ancistrodon contortrix), and the water moccasin (A. piscivorus) are widely distributed in the United States.

There are many harmless snakes which more or less resemble these "Pit Vipers," as the rattlers, moccasins, and copperheads are called. This term refers to a deep hole or pit found on the side of the head between the nostril and the eye. It is a blind sac. The much dreaded "fer-de-lance" (Lachesis lanccolata) is a crotaline snake.

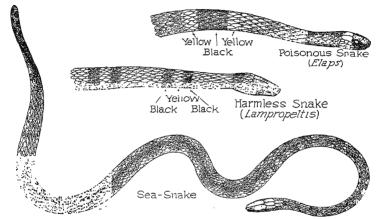


FIG. 165.—The poisonous coral snakes of the U. S., Flaps fulvius and Elaps euryxanthus, have transverse rings of black, vermillion and yellow. As differentiating these snakes from harmless ones which resemble them there are black rings bordered by two yellow ones, while with the harmless snakes a yellow ring is bordered by two black ones. The sea snake (Enhydrina species) has a rudder-like tail which is here shown twisted to one side.

Some divide the Viperidae into the Crotalinae, which possess the pit, and the Viperinae which do not have this structure. Russell's viper (*Daboia russelli*) is the best known of the Viperinae and is one of the most important poisonous snakes of India.

The poison fangs are grooved or perforated and connected with the poison glands which resemble salivary glands and may be almost an inch in length in large snakes. The tongue is slender and forked and is a tactile organ.

The jaws are remarkable for their great extensibility, not only vertically, but laterally, permitted by the ligamentous connections of the two halves of the mandible or lower jaw.

As the fangs are directed backward it is necessary for the snake when striking to open the jaws widely and bend back the neck. The fangs are then brought forward

and erected by the spheno-pterygoid muscles. The snake bite is a combination of bite and blow. The functional fangs of colubrine snakes however are not mobile.

In addition to the possession of the pit, these vipers have a more or less triangular head and in particular a single row of large scales on the under surface posterior to the vent (anus), while the harmless snakes show an elongated oval head and two rows of large ventral scales posterior to the vent.

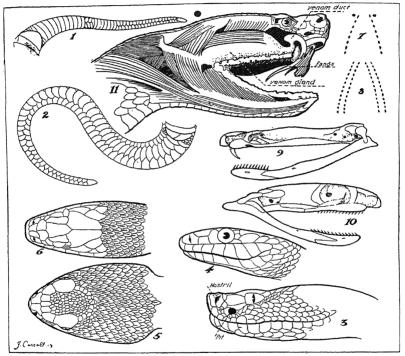


FIG. 166.—I, Single row of scales posterior to vent (poisonous snake—water moccasin); 2, double row of scales of harmless snake (*Natrix*); 3, side view of head of pit viper; 4, side view of head of harmless snake; 5, dorsal view or pit viper; 6, dorsal view of harmless snake; 7 and 9, bite puncture and skull of *Elaps*; 8 and 10, same of harmless snake; 11, poison apparatus of rattlesnake.

Snake Venom.—In examining the wound made by a snake the two punctures of the fangs indicate the bite of a poisonous snake. If these fang-puncture points are far apart it shows that a large snake, and probably one capable of injecting a greater amount of venom, has given the bite.

When a snake strikes, the fangs move from the horizontal to the erect position, the mouth being widely open. When the fangs enter, the jaws close and pressure is exerted on the poison glands so that the venom pours out.

The idea that a snake exhausts its venom when striking is not true. Colubrine snakes may bite shortly after the first attack, and inject each time a lethal dose of venom. Fresh venom varies from an almost colorless fluid to one with a brownish or greenish color. It is viscid and quickly decomposes from the varied bacterial flora it contains. A number of years ago the injection of rattlesnake venom was used in the treatment of

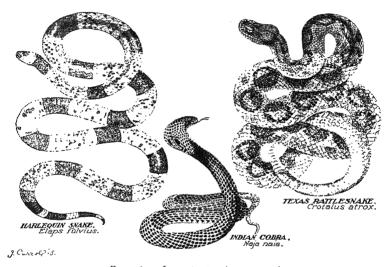


Fig. 167.—Important poisonous snakes.

epilepsy but dangerous and even fatal reactions resulted from the pathogenic anaerobes at times present in the venom of snakes. Dried venom is quite stable in the dark, and retains its toxicity for years.

The amount of venom varies with the size and condition of the snake, an adult cobra yielding about 1 cc. Acton and Knowles give the following table expressed in milligrams of desiccated venom.

Common cobra (mean yield)	317.0	mg.
Common krait (mean yield)	8.17	mg.
Banded krait (mean yield)	64.4	mg.
Russell's viner (mean vield)	108.0	mg.

They estimate the minimum lethal dose for man as 15 mg. with cobra venom and 42 mg. with the venom of Russell's viper (Daboia). The venom of the kraits is more potent, that of the very common Indian krait, Bungarus candidus, being given as 1 mg.

The cobra, after having bitten, remains attached for a short time while the Daboia strikes with the greatest rapidity and immediately releases itself.

Cobra and krait bites (colubrine snakes) produce more or less similar symptoms such as paralysis of articulation with nausea and vomiting and later paralysis of the respiratory apparatus. There is only an insignificant reaction at the point of bite.

The venom is mainly neurotoxic, causing death by paralysis of cardiac and respiratory centers. Cobra venom is also very haemolytic. This haemolysin is activated by the normal complement of the serum of the animal poisoned, the haemolysin as contained in the venom not being toxic when alone. Lecithin also has the property of activating the haemolytic substance in venom.

In rattlesnake bites (viperine snakes) there is marked pain at the site of the wound with much swelling and haemorrhagic infiltration. The swelling and petechial mottling spread up the limb from the point of entrance of the venom. Cold sweats, nausea, cardiac depression, and syncope are common. An exception to this general rule is *Crotalus terrificus*, whose venom is strongly neurotoxic, affecting vision and respiratory centres. The local effects are slight.

Rattlesnake venom is active chiefly on account of its haemorrhagin, or rather endotheliolysin, which destroys the endothelial lining of blood vessels.

The haemolytic (haemotoxic) effects of the venom of the West Indian and Central American vipers are most marked—haemorrhages from the conjunctivae and stomach occurring along with reflex vomiting. There is marked damage to the blood vessel walls, death occurring in coma in about eight hours in the absence of antivenin. Even with such treatment transfusion may be necessary. Of the American pit vipers, the rattlesnake venom is the most toxic and that of the water moccasin least so, but the necrotizing power of the latter is more marked.

Venoms may also contain proteolytic ferments which may account for the softening of muscles in snake-bite cases. The toxic effect of the venom takes place without an appreciable incubation period, hence different from true toxins.

The most venomous snakes seem to be the sea-snakes (Enhydrina). This venom is almost entirely neurotoxic.

The tiger snake of Australia is almost equally venomous and the krait (B. candidus) next. The rattlesnake is about one-fifth as venomous as the krait.

Certain venoms greatly increase the coagulability of the blood so that intravascular thromboses may occur. It is chiefly with the venoms of *Daboia* and *Bungarus* that such thromboses are likely to occur and this accounts for the almost instantaneous death which at times results from bites of such snakes, when the toxin is injected directly into a vein.

Treatment.—The non-specific treatment of snake-bite poisoning which has usually been recommended is: (1) Apply a tight ligature above the site of the bite for 20-30 minutes. The ligature, which should preferably be a rubber band, is to be applied about a single-bone extremity, not about one with two supporting bones. (2) Make deep incisions about the fang punctures and thoroughly irrigate with a strong solution of

potassium permanganate. Rogers has recommended that punctures be enlarged with a lancet and the resulting wound packed with crystals of permanganate.

Bannermann has shown that a dog bitten by a cobra cannot be saved by free incision and the rubbing in of permanganate crystals. It may, however, be saved by the immediate injection of 10 cc. of a 5% solution of permanganate, but not if two minutes has elapsed. Bites from the Daboia are fatal, however the permanganate be applied. therefore, does not consider the permanganate treatment of any practical value. Rogers thinks that Bannermann's experiments with dogs do not give a true idea of the value of permanganate because he has had success in experimenting with cats and because it has saved human lives. Chromic acid injections (1%) have also been recommended. Acton and Knowles consider potassium permanganate as unreliable and recommend subcutaneous injections of a 5% solution of gold chloride. These local injections are efficacious if used before the venom has been absorbed but they have no effect on venom taken up by the circulation. Intravenous injection of permanganate is not only without effect but is dangerous. Amaral states that the ligature will not prevent the venom from spreading and may accentuate the proteolytic and cytolytic action. In his opinion permanganate solutions in active concentrations have a deleterious action on tissues.

Internally alcohol does not seem to be of any value; in fact, many of the deaths have been attributed to excessive ingestion of whiskey. Strychnine in large, almost poisonous, doses was highly recommended in Australia, but the statistics seem to make the value of this remedy doubtful.

In an article on snake bite, N. Hamilton Fairley (1934) states that early free excision combined with mechanical suction is the only method of local treatment likely to be successful in body bites. Immediate application of a ligature and free excision (3 by 3 cm.), down to the muscles, was the only effective local treatment in sheep bitten by tiger snakes. He refers to the work of Crimmins (1927), advocating ligature and incision combined with suction by a breast pump, and that of Jackson and Githens (1931), in which incision of the wound bite, combined with suction by a Bier's apparatus and irrigation with saline, is recommended. Fairley regards these methods as useful accessories to ligature, incision and excision. As regards the use of permanganate, Raymond Ditmars (Cecil's Medicine) states "Nothing could be more foreign to the treatment of snake bite than such practice." For local treatment he advocates deep incision and forced suction. Cauterization should never be used.

Antivenins.—The active agents of snake venoms may be either of the nature of haemorrhagins, neurotoxins, or fibrin ferments. In colubrine snakes the neurotoxin vastly predominates whereas with the viperines it is the haemorrhagin. Certain Australian snakes contain all three bodies in about equal proportion, whereas with the rattlesnakes of America it is almost entirely the haemorrhagin which causes the poisoning. The Elaps of Florida is a colubrine snake and its venom is neurotoxic in nature.

The cause of death in colubrine snake bites is chiefly from paralysis of the respiratory centers whereas with the pit vipers it is chiefly from haemorrhages in the vital organs. Antitoxins have been prepared against both viperine and colubrine venoms and these are specific; thus a colubrine antivenin will not be of value against a viperine bite. Antivenins should be administered either intravenously or intramuscularly. The amounts recommended for injection to neutralize a fatal dose of snake poison vary from 100 to 300 cc. of the antivenin serum. There is no accurate method of standardization.

When Calmette (1894) first produced antivenin the idea prevailed that it was useful for any snake venom, a view soon found to be untenable. There are now institutes in many parts of the world where antivenins are made to combat the local venoms; thus in the U. S., we have the Antivenine Institute of America which produces an antivenin for rattlesnake, copperhead and water moccasin venom. These venoms are chiefly haemorrhagic. Previously, the toxicity of some venoms made the immunization of horses precarious, but methods of detoxication are now being used which are more successful. Both monovalent and polyvalent sera are produced. Often, when it is impossible to determine the species of the offending snake, a polyvalent serum is indicated. Antivenins are given either intramuscularly or intravenously. With highly poisonous venoms intravenous therapy is indicated. Fairley emphasizes that dosage is in inverse proportion to body weight, so that children may require several times the amount of serum sufficient for a heavier adult. This is connected with the natural neutralizing power of the blood stream. A large individual, having more blood to partially neutralize venom than a smaller person or a child, requires less antivenin. Owing to varying strengths of antivenins (concentration methods) one should depend for dosage on the instructions accompanying the product. Besides the local and specific treatment for snake bite one should put the patient at rest physically and mentally, as psychical shock is an important matter with some snake bite patients. Fairley also recommends black coffee or caffein. Avoid strychnine and alcohol, and in particular morphine.

# LIZARDS

Lizards are non-poisonous, with the exception of the Gila monster (Heloderma suspectum). It is chiefly found in Arizona and New Mexico (Gila river valley). It is about two feet long, heavily built and covered with small tubercles. The name monster is most applicable. The poison fangs are in the lower jaw, and the bite of this apparently sluggish creature may cause death. When aroused, it is very vicious, and it is as difficult to open the closed jaws as in the case of a bulldog. It deposits its parchment-like eggs in the sand of the desert.

## CHAPTER XXV

# POISONOUS ARTHROPODS, FISH AND COELENTERATES

## VENOMOUS ARTHROPODS

Spiders.—Spiders belong to the class Arachnida, order Araneida. There are numerous families, divided into various genera. As a rule spiders secrete a venom which is capable of poisoning the small animals used as food, but it is only in rare instances that the venom is poisonous for man. Individual idiosyncrasies may make one person susceptible to spider or other arthropod bites whereas others do not suffer.

Reports of illness following spider bites are very rare and many of these are due to secondary infections with pyogenic bacteria.

The dread of spiders is probably connected with attributing the hysteria of the Middle Ages, or tarantism, to the bite of *Lycosa tarantula*. As a matter of fact the bite of this spider produces only a localized erythema without general symptoms.

Experiments have shown that most of the common spiders not only are unwilling to bite but, even when almost forced to do so, are unable to penetrate other than the most delicate human skin. Even then the bite has only the effect of a pin prick.

In America we apply the term tarantula to a large, dark, hairy, ferocious-looking spider of the family of Aviculariidae. The American tarantula, *Eurypelma hentzii*, is capable of killing very small animals but it is believed to be able to inflict only mechanical injury on man.

Certain species of the genus *Latrodectus* produce systemic symptoms rather than local ones. The bite of *L. kapito*, of New Zealand, is stated to slow the pulse and respiration and produce tetanoid manifestations.

Latrodectus.—In the U. S. a spider, L. mactans, often called the "black widow" or "hourglass" spider, has been held responsible for symptoms of poisoning in about 400 cases, with a record of 16 deaths.

These spiders have been reported from many states, particularly Western and Southern ones, with about half of the cases from California. In his book on entomology, Herms gives an excellent description of this spider. The female is about an inch in length—the male is much smaller. The globose abdomen of the female stands out like a beautiful highly polished black pearl. She is exceedingly active and most aggressive. An interesting fact is her killing the male after he has served the ends of species preservation.

It has been stated that the more husbands she has disposed of the greater the virulence of her venom. This spider is usually found in old out-buildings, chiefly under

privy seats, or dry cracks in brick or concrete work supporting such buildings. They also can be found in new houses and occupied ones, even in occupied beds. The web is rather coarse and does not show the regular symmetry of many spider webs. The egg cocoon is attached to the web, and any attempt to dislodge it is resented by the alert mother. There are more than 100 rather large eggs in the cocoon, which is spun during the summer. The small grey spiderlings, which hatch out in about a month, are very active. After several molts, at each of which there is increasingly darker color, we have

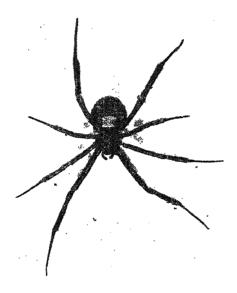


Fig. 168.—Latrodectus mactans, female. (Black widow.) Ventral surface showing orange-red hour-glass spot. (Original.) Approximately actual size.

after a number of months (and generally in the spring), a mature female with the deep orange spots on the ventral abdomen which enable us to identify this species. The spots vary from a striking resemblance to an hourglass, with the spots apposed at their apices, to an arrangement like a Maltese cross with four spots at right angles. Again there may be only one spot. Cases of bites generally occur in the summer and autumn.

W. H. Chapman (1936) notes the severe systemic symptoms of four cases observed by him—rather insignificant pain at the site of the bite (which may not be manifest), to be followed in about 30 minutes by marked pain in various parts of the body, particularly of the abdomen, when the rigid muscles make one suspect "acute abdomen."

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The pain is excruciating and scarcely responds to morphine. The pulse is slow and the respiration embarrassed. The temperature may be raised and the leukocytes increased. There may be a macular rash. Spasm of the back and thigh muscles may be noted. Hot packs seem to be indicated and alcohol to be condemned. Gilbert and Stewart (1935) recommend intramuscular or even intravenous injections of calcium gluconate (10 cc. of a 10% solution).

A species of Glyptocranium, G. gasteracanthoides, found in Peru, gives rise by its bite to gangrenous lesions, haematuria, etc. The bite may be fatal.

Scorpions.—These arachnids belong to the order Scorpionida. The scorpions of temperate climates are usually small but those of the tropics may attain very large size, even 7 inches in length. The last abdominal segment terminates in a ventrally curved spine. This segment carries the poison glands.

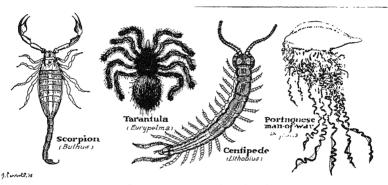


Fig. 169.—Poisonous arthropods and coelenterates.

Scorpions have formidable claws or pedipalps, with which they seize their prey and then by a downward movement of the tail-like abdomen they pierce the prey with the spine and thus introduce their venom. The poison of some of the large scorpions, as Bulhus quinquestriatus, seems to resemble in action that of the cobra venom. Although the larger scorpions are particularly to be dreaded, especially where young children have been bitten (mortality of bites of B. quinquestriatus in young children practically 50%), the effects of the bites of the small scorpions found in the Southern U. S. and California are probably never fatal although they may be quite painful and produce slight general symptoms.

Myriapods.—These arthropods are divided into the orders, Diplopoda, or millipedes, and Chilopoda, or centipedes. Millipedes have a more cylindrical body than centipedes and, with the exception of the appendages coming from the most anterior somites, have two pairs of legs to each segment, whereas the centipedes have only one pair to each segment.

It is generally accepted that millipedes are harmless. Centipedes have poison glands at the base of the first pair of legs. The legs terminate in a powerful claw, at the tip of which is the opening for the expulsion of the venom. The small centipedes which are found in temperate climates rarely give rise to more than local symptoms, but the large tropical ones, as for instance Scolopendra gigantea, which may be 10 to 12 inches long, may cause death in children by their sting. They give rise to necrotic local lesions at the sites of the two punctures, and, in addition, may produce general symptoms of vomiting, headache, fever and even coma.

Bees, Wasps and Ants.—These arthropods belong to the order Hymenoptera of the class Insecta. The venom of bees is ejected through the sting which is at the end of the abdomen. In addition to the formic acid there is also a neurotoxin in the venom. When a bee stings, the biting parts are left in the wound to continue by muscular action to force out the contained venom into the tissues of the victim.

As a rule the effects of a bee sting are entirely local, but cases have been reported of general symptoms ensuing, such as fever, dizziness, dyspnoea and urticarial lesions.

The bumble bee differs from the honey bee in that the sting is not cast off when stinging. Hornets and wasps have a well developed sting and are more dreaded for their sting effect than bees.

Ants.—In temperate regions ants rarely are considered as producing injury but in the tropics there are large formidable species which may not only cause local irritation but even produce general symptoms of nervous system involvement.

In the Philippines the ants are prominent factors in destroying house fly larvae so that in this way they are of great assistance to man.

Caterpillars.—Certain caterpillars, of various families of Lepidoptera (moths), have hairs with poison gland cells, which irritate the skin, producing a more or less extensive rash. The caterpillars of the brown-tail moth have hairs which when shed and coming in contact with the skin cause the "brown-tail rash." Allergic reactions may be produced by venoms, scales, or other products of anthropods which may not generally be recognized as poisonous—eczemas, coryza or asthma.

Beetles.—The best known of the urticating (vesicating) beetles is Lylla vesicatoria, the Spanish fly, the source of cantharadin. There are other species of blister beetles.

Cone-nosed Bugs.—These have been discussed on page 539 (Reduviidae). The bites of certain species of *Reduvius* (kissing bug) or *Triatoma* may cause nausea, acceleration of pulse and respiration, and urticaria. Species of *Rasahus* (Corsairs) are noted for the severity of their bite, which is often attended with cellulitis, followed by bacterial infection and possible septicaemia.

#### Poisonous Fish

Fish Poisonous as Food.—Illness produced by eating decomposed fish, whether in the natural state or canned, belongs to the general problem of

food poisoning. There are, however, certain fish whose meat is poisonous when eaten in a perfectly fresh state.

This may be connected with certain epidemic diseases among fish ordinarily good food. Various bacterial organisms have been isolated from such fish, and the poisonous effects have been attributed to various ptomaines elaborated by these toxicogenic organisms. Most of the organisms isolated from diseased fish have belonged to the colon or proteus groups. Cases have been reported of botulism-like poisoning arising from the eating of insufficiently salted fish. These cases were probably due to the

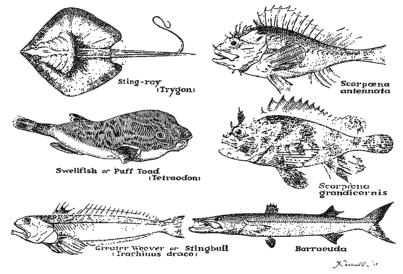


Fig. 170.-Poisonous fishes.

development of a soluble toxin by Cl. botulinus, as such fish when cooked lost their toxicity. The toxin of Cl. botulinus is destroyed by heat, whereas that due to the Gärtner, or ordinary food poisoning organism, withstands ordinary cooking temperatures. This lish poisoning by bacterial products is designated ichthyotoxismus.

The only two important animal parasite infections with which the eating of fish is connected are: (r) Diphyllobothrium latum and (2) Clonorchis sinensis. The broad Russian tape-worm is a rather common parasite of man in the Baltic provinces and comes from eating insufficiently salted pike and other fish infected with this larval tape-worm. The liver fluke disease of China and Japan is caused by eating various raw or insufficiently cooked fresh water fish. These fish are the secondary

intermediate hosts, the primary ones being molluscs. A very small fluke of Japan, *Metagonimus yokogawai*, is transmitted by the ingestion of certain goldfish.

There are certain fish whose meat is poisonous when there is no question of decomposition or disease in the fish. The best known instance is with certain species of the genus Tetrodon. The illness produced by the eating of this fish is usually termed fuguismus, the Japanese designating such fish by the term "fugu." The poisonous principles seem to exist chiefly in the ovaries and testes, the eating of even one roe of such fish bringing on serious illness in a few minutes or possibly death in a few hours. It has been stated that after careful removal of all genital and alimentary tract organs these fish may be eaten without harm. The poisonous principle has a physiological action somewhat like curare, and is thermo-stable. Such fish have been used to commit suicide. The porcupine fishes or Diodontidae are considered as poisonous. These fishes together with the Tetraodontidae, or broad-nosed puffers, are unsightly in appearance. Among seamen they are generally designated puff toads since they become distended with air as they are drawn out of the water. It is well recognized that certain of these fish which may fail to cause poisoning at one time may do so at another time and it is particularly noted that poisoning effects occur at the time of spawning.

In the tropics fish which may ordinarily be safe as food become poisonous as a result of feeding on certain poisonous medusae and corals.

This is probably true of the barracuda, which is eaten with impunity at most times. Yet undoubted cases of poisoning with this fish have occurred. It has also been suggested that the barracuda may be poisonous at certain times in its life, for example, during spawning, or that it is subject to a more rapid decomposition at such times.

To test reports of such poisoning, Bloedorn and Hakansson (1926) reported the eating of barracuda with safety on several occasions. This fish was one of those most commonly caught in the West Indian waters surrounding Puerto Rico. The fish, eaten on several occasions, varied from 8 to 15 pounds and one weighed 26 pounds. One of the warnings from local fishermen was that very large barracudas were not safe. Fish poisoning in the tropics from barracuda or other edible fish is probably most often due to commencing putrefaction.

There are certain species of the herring family which have a bad reputation. Among these are two species of Meletta. In New Caledonia, M. venenosa causes painful cramps of the body with dyspnoea, cyanosis, cold sweats and dilated pupils and at times death. M. theissa of the West Indies is also a very poisonous fish.

Herre describes in the Philippine Journal of Science for October, 1924, 60 species of poisonous and worthless fishes, of the order Plectognathi.

Fish Which Poison by Their Sting or Bite.—Fish of the genus Muraena have well developed teeth which are in relation to a poison sac which secretes a venom which is introduced into the wound made by the bite. There are also various rays which are well known all over the world as capable of inflicting wounds.

In the sting rays (Trygonidae) the tail is armed on the upper side with a barbed spine which in some species is connected with a poison apparatus. Some of these sting rays when wounding a person who may step on them while wading in the water may at the same time inoculate tetanus bacilli which are particularly dangerous because of the character of the deep punctured wound. In the electric rays (Torpedinidae) the dorsal surface is electrically positive and the ventral one negative. To receive a shock one must communicate with the Torpedo species at two distinct points. Some of these electric rays are capable of temporarily paralyzing the arm of a man.

Two of the best known poisonous fish are *Trachimus draco* and *Scorpaena scropha*. The flesh of these fish is wholesome as food. *T. draco* is like a trout in appearance and has blue and brown stripes. It has a grooved spine passing through each of its gill covers which is connected with a poison gland. There is also a poison apparatus connected with the dorsal fin. *S. scropha* is an ugly red fish with large head and prominent fins. The French fisherman call it "le diable." The poison apparatus is connected with the first three rays of the dorsal fin.

Persons in bathing who strike against these fins are more apt to be wounded than the fishermen who handle the fish with caution. Following the wounding a person experiences stabbing pains in the affected part. A sensation of suffocation follows and the victim may become delirious. At times collapse and death result. At the site of the wound we first have an erythematous area which later becomes black and may become gangrenous. As the poison rapidly enters the general circulation treatment similar to the local treatment of a snake bite is called for. These fish seem to be more dangerous during the spawning period.

## MOLLUSCS AND DISEASE

The importance of disease transmission by snails has been taken up under the helminthic infections caused by flukes.

Oysters. There are more than 100 species of true oysters (Ostrea), exclusive of various allied forms, such as the pearl oyster or the window-pane oyster. Oysters belong to the class Lamellibranchia and the two most important edible species are O. edulis, the European oyster and O. virginica, the Americo-Canadian one. The question often arises as to the edibility of oysters which are green in color. This color is considered desirable in certain European countries, and is produced in France by feeding the oysters a diatom, Navicula ostrearia. The green pigment is present in the gills and palps. Such greening may be natural. Sometimes there is a green color of the body of the oyster due to copper.

Typhoid fever and oysters.—There have been numerous outbreaks of typhoid fever in both the United States and England for which the eating of contaminated oysters was responsible. Oysters may be contaminated when growing in sewage-contaminated beds, but in the studies of typhoid infection from eating raw oysters the blame has been placed on polluted

water used for floating oysters. If the water in which the oysters are placed for storage (floating) is pure, such a process makes the oyster safer. At present, floating in chlorinated sea water is of particular sanitary advantage. Outbreaks of typhoid fever, as for instance that of the N. Y. epidemic of 1924–1925, when more than 100 deaths from typhoid fever were recorded, have led to official regulation. In uncontaminated salt water, studies have shown that the oyster gets rid of the typhoid organism in from two to three weeks.

It is probable that the only ground for considering a sound oyster as capable of causing food poisoning is from its effect on individuals with an idiosyncrasy to shell fish—and such idiosyncrasy seems not uncommon.

Mussels.—These also belong to the class Lamellibranchia. Mussels are widely distributed, and in Europe form an important article of food (the edible mussel, Myillus edulis). They seem to thrive better in saline waters which have a somewhat lesser salt content than sea-water. Fresh water mussels have an economic value in the use of their shells for button-making. Besides ill effects related to idiosyncrasy, we may have poisoning leading to paralysis or even death. In California (1927), there were reported 192 cases of mussel poisoning with 6 deaths. The symptoms developed in 10 to 20 minutes after ingestion. The toxin seems to be thermostable and is absent except during the spawning season (June through September).

#### Poisonous Coelenterates

In the phylum Coelenterata we find animals of very simple structure, only the sponges and protozoa having a more lowly type. It is customary to distinguish two morphological types of coelenterates, the polyp and the medusa.

The Polyp.—The best example of a polyp is a sea anemone.

Quite interesting in the study of immunity is the constant association of an anemone with certain hermit crabs. The anemone covers the soft tail-end of the crab, thus protecting the crab from attacks by its enemies. The mouths of the two animals are in close juxtaposition so that the food of the crab is shared with the anemone. This crab acquires an immunity to the poison of the anemone, probably as the result of frequent ingestion of fragments of anemone. Other crabs are very sensitive to the anemone poison, suffering paralysis and death. The poison of certain anemones may even harm other anemones.

A condition known as "la maladie des plongeurs" occurs among the sponge fishermen of the Mediterranean. This is due to stinging by anemones and is characterized by marked itching, burning and erythema. In some cases the skin of the affected area becomes necrotic and sloughs off leaving an ulcer.

The Jellyfish (Medusa).—This umbrella-like coelenterate has tentacles which hang down from the margin of the jellyfish.

As a rule jelly fishes are harmless but certain species produce unpleasant or even serious effects by their sting. Cases of lesions following contact with unspecified "jelly-fish" have been reported by Allen (1920) and Stewart (1922). The local rash in Allen's case was followed by profuse weeping eczema, by aphonia, and by laryngitis lasting for four weeks. Aoki (1922, 1923) portrays the severe effects, besides urticaria, of stinging by Olindioides formosu—shock, acute cardiac distress, dyspnoea, muscle pains, and as a possible sequela emaciation.

In the Mediterranean a jelly fish *Rhizostoma pulini* produces oedema and urticarial eruptions as the result of its sting. In many parts of the tropics jelly fishes are found which give rise to quite serious symptoms. In the Philippines there are certain species of jelly fishes which cause serious illness, although as a rule one experiences no discomfort from coming in contact with many other species while swimming in the waters of that part of the world.

According to Light, the species of *Dactylometra*, called "fosforo" by the natives, is the most dangerous one there encountered. It has long ribbon-like oral lappets and 24 slender white marginal tentacles. In this the sting is inflicted by nematocyst batteries in the four long ribbon-like oral palps. *Lobonema*, called by the natives "lanterna," is of large size, white or white and purple in color, and stings by the long filaments which arise from the mouth arms. I have treated a number of cases of jelly fish stinging in the Philippines which presented symptoms ranging from a mild crythema to those showing marked congestion of the respiratory tract and other general symptoms.

Old has described these symptoms very accurately and notes the following:

The symptoms appear in from ten to sixty minutes with marked hysterical manifestations, incessant cough and coryzal signs. Light believes that the cases described by Old were due to stinging by Dactylometra.

Wade describes his own experience with a jelly-fish sting while swimming in Manila Bay. The tentacles became wrapped about the upper arm and stinging was instantaneous as the tentacles did not cling. The poison did not reach the conjunctival or other mucous membrane. There was at once a sensation of burning in the area of contact, but it was 15 minutes before other symptoms appeared. There was pain in the loins and also in the scrotum. This was followed by a curious restlessness and weakness, then a sense of constriction in the throat, with chest discomfort and then coryza and lachrymation. The symptoms abated and within an hour there only remained weakness and soreness of the

bronchi. A vesicular dermatitis appeared on the arm and the traces of the sting had not disappeared after 2 or 3 weeks. Other cases have been reported associated with feeble heart action and semi-conscious states. There is always to be considered the possibility of one's drowning when in the emotional or semi-conscious state. Wade describes a death in a robust Filipino who was stung on the leg. His companions were only a few yards away, but by the time they had reached him he had collapsed and was gasping and livid, and was dead a few moments later. It was at first thought he had been bitten by a sea-snake but there was no mark on the leg, except conspicuous purplish discoloration. On autopsy he showed status lymphaticus with persistent thymus, acute congestion of the viscera and oedema of the lungs.

The Portuguese man-of-war (*Physalia*) has long locomotive tentacles which stretch out from 30 to 50 feet as the animal is blown along by its pearly purple crested bladder-like float or sail. The thread cells are capable of inflicting rather painful stings when handled without a knowlege of the effect of coming in contact with these thread cells.

# PART IV

# CLINICAL AND PATHOLOGICAL EXAMINATIONS OF THE VARIOUS BODY FLUIDS AND ORGANS

# CHAPTER XXVI

# DIAGNOSIS OF INFECTIONS OF THE OCULAR REGION

It is advisable before taking material for cultures or smears to cleanse the nasal area of the eyelids, especially about the caruncles, with sterile salt solution. Then, by gently pressing on the lids, we may be able to get pure cultures of the organism causing the infection.

A small particle of sterile cotton, wound on a toothpick, with the aid of a sterile forceps, makes an excellent swab for obtaining material for smears, and may be used to make cultures on agar plates before making smears on slide or cover glass.

When there is a considerable discharge, a capillary pipette, with a rubber bulb, may be used to draw up sufficient material for cultures and smears. Be sure to round off the end of the pipette in the flame and not to use a very fine capillary tube.

Another good method of obtaining material for culture from the eye is to instil a few drops of broth and then recover it by loopfuls for inoculating media.

In conjunctival cultures, plates of glycerin agar, blood agar, or agar plates smeared with blood are to be preferred, as the *Gonococcus* and Koch-Weeks bacillus will grow only on blood or hydrocele agar. The diphtheria and xerosis bacilli grow well on glycerin agar.

Normally, we may find in the region of the caruncles various skin organisms, especially staphylococci giving white colonies.

The xerosis bacillus and white staphylococci may be considered normal findings in the conjuctival sac. Streptococci and pneumococci also have been reported from apparently normal conjunctival secretions. The *C. xerosis* must not be accepted as explaining an infection unless other factors have been eliminated. The true diphtheria bacillus, which the xerosis so much resembles, may cause a pseudomembranous inflammation.

The Staphylococcus is as a rule the cause of phlyctenular conjunctivitis. In addition to the white Staphylococcus, the Streptococcus may be present when inflammation of the nasal duct exists. The Streptococcus is at times responsible for a pseudomembranous conjunctivitis.

It is now recognized as advisable to make an examination for the *Pneumococcus* before performing operations on the eye as serious results may follow if the *Pneumococcus* be present. It is the organism most frequently responsible for infections after cataract operations. It is frequently found in dacryocystitis and, in the case of traumatism, may bring about panophthalmitis.

The *Pneumococcus* is a fairly common cause of scrpiginous corneal ulcerations for which active treatment is necessary. Corneal ulcerations are not apt to appear even with a pneumococcal conjunctivitis unless there be an injury of the epithelium.

The pyocyaneus bacillus may cause severe purulent keratitis as well as conjunctivitis. The pyocyaneus toxin appears to be a factor in the production of the lesions observed.

The *Gonococcus* and the Koch-Weeks bacillus are usually responsible for the very acute cases of conjunctivitis. Both these organisms are characteristically intracellular and are Gram-negative.

In a gonorrhoeal ophthalmia the secretion is much more abundant and there is an absence of contaminating organisms, the reverse of infection with the confusing *N. catarrhalis*. As a matter of fact, large numbers of *N. catarrhalis* may be present in the conjunctival secretion with only slight irritation being observable. Gonorrhoeal ophthalmia seems to be remarkably infrequent in the female children with gonorrhoeal vulvo-vaginitis. Gonorrhoea, frequently long after the acute urethritis, may be responsible for iritis in men (extremely rare in women).

Iritis and iridocyclitis are most often due to a focal infection, but, in the presence of a positive Wassermann reaction, a syphilitic origin must be thought of.

Conjunctivitis in the course of epidemic cerebrospinal meningitis and even panophthalmitis have been found to be due to the *Meningococcus*.

The diplobacillus of Morax and Axenfeld is more common in chronic, rather dry affections of the conjunctiva, chiefly involving the internal angle and showing a morning accumulation of the secretion. The bacilli are found in twos, more rarely in short chains. They are generally free but may be found in phagocytic cells. They resemble Friedländer's bacillus morphologically but do not have capsules.

In cases of ozaena with involvement of the nasal ducts Friedländer's bacillus may be found.

Even in cases without ozaena, capsulated, Gram-negative bacilli of the Friedländer group have been frequently reported in conjunctival inflammation and in dacryocystitis as well.

The nodules of the eyebrows give the most convenient area from which to take material in the diagnosis of leprosy, either the fluid expressed after scraping or a piece of tissue cut into sections. Conjunctival ulceration in leprosy may show abundant bacilli as is also true of corneal ulceration.

Ordinarily it is impossible to find tubercle bacilli in tuberculous conjunctival discharges.

The discharge from a tuberculous dacryocystitis may show them satisfactorily. Animal inoculation is preferable in the diagnosis of ocular tuberculosis. The *Pneumo-coccus* is, however, the most important organism in dacryocystitis—rarely the colon bacillus.

Choroido-retinitis, uveitis and other inflammatory lesions are frequently associated with a strongly positive tuberculin reaction and are attributed by some to an allergic reaction to products liberated from tuberculous lesions in other organs.

P. tularensis.—Wherry has reported cases of ulcerative conjunctivitis with lymphadenitis of cervical glands, fever and marked prostration, due to infection with this organism.

In keratomycosis the cause has been ascribed to Aspergillus fumigatus.

Trachoma is now believed to be due to a filtrable virus. The trachoma bodies are a type of cellular inclusion body.

A conjunctival irritation may be allergic in origin, and in such cases the smear from the secretion often shows the presence of eosinophiles.

Animal parasites.—The larval form of Taenia solium (Cysticercus cellulosae) has a predilection for eye as well as brain. It is usually situated beneath the retina.

The question as to the nature of the so-called ophthalmic flukes is taken up under trematodes. *Echinococcus* cysts have been reported in the orbit.

The adult Loa loa tends at times to appear under the conjunctiva or in the subcutaneous tissue of the eveilds.

The larvae of *Onchocerca volvulus* may be present (in chronic cases) in the comea and other tissues of the eye, causing a characteristic punctate keratitis, iritis, and eventually blindness.

Fly larvae have been reported from the conjunctival sacs in the helpless sick, species of larval sarcophagids having been reported as invading the conjunctival region in purulent ophthalmias.

Demodex may cause an obstinate blepharitis.

For xerophthalmia and night blindness, see p. 795.

## CHAPTER XXVII

# DIAGNOSIS OF INFECTIONS OF THE MOUTH AND PHARYNX AND OF THE NOSE AND EAR

NORMALLY the mouth and pharynx contain enormous numbers of bacteria of many kinds and frequently various protozoa and fungi. Many of these organisms are harmless saprophytes, some are potential pathogens, and sometimes virulent bacteria are harbored which the individual can resist sufficiently to prevent the development of clinical infection, but which may cause disease in others.

Staphylococci (aureus and albus), streptococci of different types, diphtheroid bacilli, pneumococci, sarcinae, colon and other Gram-negative bacilli are commonly found. Friedländer's bacillus, proteus, *M. catarrhalis*, leptothrix, and various yeasts and molds occur. Spirilla and spirochaetes are almost constantly present. In addition various organisms occur which have not yet been carefully studied and classified.

Bacteriological studies of infections in this region are, therefore, difficult, and their interpretation requires considerable experience. Doubtless some of the pathogenic organisms present are in a dissociated phase, and their significance will be better understood when more is known about the variability of different species.

In certain conditions, however, bacteriological studies are of value.

Material is obtained with a sterile, cotton-tipped wire or wood applicator. Sterile forceps may be convenient for obtaining particles of membrane. For making smears from around the gingival margins a sterile tooth pick is useful. Antiscptic gargles or mouth washes should not be used for several hours before taking a culture.

Diphtheria.—In individuals with clinical diphtheria, the diphtheria bacilli are usually so abundant and unmistakable morphologically that their demonstration by culture and smear is practically diagnostic. However, in the absence of typical lesions, morphologically typical bacilli may be present which may be avirulent, or the individual may be a healthy carrier of virulent organisms. This distinction is, of course, of great importance. Methods for making these tests are discussed in the section on the diphtheria bacillus. Diphtheroid bacilli, which are almost always present in the normal throat, can usually be differentiated morphologically.

Streptococcus sore throat.—Smears from the inflamed area usually show large numbers of streptococci. Cultures should be made by streaking a blood agar plate on which haemolytic streptococci are easily distinguished from other types. If haemolytic streptococci are present in large numbers, the culture has diagnostic value. Haemolytic streptococci are also constantly present in the angina of scarlet fever. However, they are present in smaller numbers in about 10% of normal individuals, and the interpretation of their presence in small numbers is difficult. The part which these carriers play in the bronchopneumonias following measles and influenza is not clear, but it would seem that individuals harboring them tend to develop bronchopneumonia, and are the chief factors in spreading this streptococcus infection to other persons.

Vincent's infections.—Ulcerative lesions of the mucous membranes associated with the fusiform bacilli and spirochaetes described by Vincent contain enormous numbers of these organisms, which can be demonstrated readily in smears. In making these smears it is important to obtain material from the depths of the ulcer. Being anaerobic organisms, they are more numerous under the membrane. If only a few are found, one should be skeptical about their etiological significance, for they are commonly present in the normal mouth, especially around the teeth. Although it is possible to obtain cultures by special methods, this procedure is not feasible for diagnosis. (See section on fusospirochaetosis.)

The bacteriological diagnosis of atypical streptococcus and Vincent's infections is not always simple. Diphtheria ulcerations may be secondarily infected with these organisms. Since the treatment of these conditions is entirely different, all of these organisms should be searched for, and considerable judgment and clinical knowledge is necessary in interpreting the bacteriological findings. In addition, streptococci, or fusiform bacilli and spirochaetes, may be found in lesions of the mucous membrane that are due to other causes, such as leukaemia and agranulocytic angina. A blood examination may be necessary to exclude these conditions. Atypical carcinomatous ulcerations can be identified by biopsy if necessary.

In syphilitic ulcers the Treponema pallidum may be demonstrated by dark-field illumination, but the presence of morphologically similar spirochaetes in the normal mouth makes this procedure useless for diagnosis. The Kahn or Wassermann test, however, is practically always positive when there are lesions in the mouth. The Treponema pallidum does not stain by the ordinary methylene blue or carbol fuchsin stains which serve to demonstrate the spirochaetes of Vincent.

Tonsillitis may be caused by different species of bacteria. Staphylococci, streptococci and occasionally influenza bacilli may cause severe inflammation. Aside from acute infections the tonsils may chronically harbor organisms in the depths of the crypts and in their substance, in which case they may act as a focus of infection (see Chap. XL). Tubercle bacilli have been found in sections of diseased tonsils.

Meningococci are found in the nasopharynx in cases of epidemic meningitis, and in many contacts. In addition an appreciable number of normal individuals have been shown to be chronic carriers. For the demonstration, cultures should be taken from behind and above the soft palate with a bent wire swab, and the material should be immediately inoculated on blood or serum agar and quickly put in the thermostat.

The thrush fungus, Syringospora albicans (Monilia albicans), causes the development of firm, hard, creamy white patches on the mucous membrane. The fungus may be easily demonstrated by mounting a bit of the membrane in 10% sodium hydrate, or by crushing and staining by Gram's method. The filaments are segmented and branching, and round or oval spores are seen between the filaments and attached to them in short budding chains. Other monilia may be found in sprue ulcerations about the tongue or buccal mucosa, and also in the faeces.

Actinomycosis may develop about a carious tooth, and the finding of the ray fungus in the yellow granules from the pus establishes the diagnosis.

Amoebae and flagellates have been reported from the mouth. For a time Endamoeba gingivalis (E. buccalis) was considered the exciting cause of pyorrhoea alveolaris, this organism being frequently obtained from scrapings about affected teeth or in the contents of root abscesses. At present they are regarded as of no importance. In the remarkable disease "halzoun," flukes have been found to be the cause of the asphyxia.

In the tropics, round worms (Ascaris) may be vomited up and, lodging in the pharynx, may have to be extracted.

During the campaign of Napoleon in Egypt many cases of leech involvement of the nasal and buccal cavities were noted. The parasite was the *Limnatis milotica* which gained access to the upper pharynx through drinking water from springs and pools. Many such cases continue to be reported from the Mediterranean basin.

#### NOSE

In taking material from the nasal cavities for bacteriological examination, it is well to wash about the alae with sterile water, then have the patient blow his nose on a piece of sterile gauze and take the material for culture or smear from this. If the material is purulent and located at some ulcerating spot, it is best to use a speculum, and either touch the spot with a sterile swab or use a capillary bulb pipette with a slight bend at the end.

In general, the bacteria found in the nose and middle ear are like those in the throat. Normally one finds chiefly white staphylococcus colonies and colonies of short-chain streptococci, occasionally *G. tetragena*, the xerosis bacillus and Hoffmann's bacillus.

Biscuit-shaped diplococci, both Gram-negative and positive, are to be found either normally or in cases of coryza. N. catarrhalis has probably been frequently reported as the Meningococcus. Still, the Meningococcus, originating most probably in the posterior pharynx, has been found in the nasal secretion of patients with cerebrospinal meningitis. H. influenzae (Pfeiffer) and the Pneumococcus have also been frequently found in cultures from the nasal secretions. The cause of the contagious type of coryza is a filtrable virus.

In some cases of ozaena we may find an organism of the Friedländer type in pure culture.

Diphtheria involving the nasal cavity must always be kept in mind, and in quarantine investigations the examinations of the nasal secretions culturally should be a part of the routine.

The tubercle bacillus may be found in nasal ulcerations; it is, however, present only in exceedingly small numbers. On the other hand, one of the best diagnostic procedures in leprosy is to examine repeatedly smears from nasal mucous membranes for the *M. leprae*. In such ulcerations the bacilli are found in the greatest profusion. Rarely glanders may cause ulcerations.

Proteus vulgaris is frequently responsible for the production of foul odors in nasal discharges but does not seem to produce inflammatory conditions of the nasal mucosa. It simply decomposes the discharges. Various fungi have been reported from the nose, but in such a region the strictest conservatism in interpretation should be observed.

A fungus-like organism (*Rhinosporidium*) has been reported in cases of nasal polyp. So many degenerative changes in epithelial cells resemble protozoal forms that such findings require ample confirmation.

The larval form of *Linguatula serrata* is a rare parasite of the nasal cavities; it is not infrequent, however, in the nostrils of dogs.

Various fly larvae are far more common, and the "screw-worm," the larva of the *Cochliomyia americana*, is common in certain parts of tropical America, and may cause death by the effects of its burrowing.

The larvae of *Sarcophaga* have in particular been found in the nasal cavities of children. Myriapods, while of very little importance elsewhere, have been reported more than 30 times from the nasal fossae.

#### EAR

The middle ear is normally free of bacteria, but in affections of the throat, as with streptococci, pneumococci, and diphtheria bacilli, these organisms may infect it by way of the Eustachian tube.

Otitis media.—In a study of the bacteriology of otitis media, in 277 cases, Libman and Celler found streptococci present alone in 81%, Streptococcus mucosus in 10% and the Pneumococcus in 8%; Staphylococcus, pyocyaneus and proteus bacilli have also been found. Mixed infections are common. A Streptococcus of the haemolytic group was an important organism in the mastoid infections so frequent during the World War.

Streptococci are the organisms which most often cause sinus thrombosis and brain abscess. The influenza bacillus of Pfeiffer has been reported as a cause of acute otitis media.

Non-virulent diphtheroid bacilli are not infrequently obtained in cultures from ear discharges. Meningococci may cause otitis media.

Other organisms which have been isolated from middle-ear or mastoid discharges are E. coli, N. catarrhalis, G. tetragena and Friedländer's bacillus.

#### 610 INFECTIONS OF THE MOUTH, PHARYNX, NOSE AND EAR

E. typhosa may be found in middle-ear discharges of persons who have had an attack of typhoid fever.

The moulds are of greater importance in affections of the external auditory canal than the bacteria. The cerumen seems to make a good culture medium so that various species of Aspergillus, Mucor, etc., develop and may obstruct the canal. These infections are often introduced by the patient's finger. Various mites and fly larvae have been reported from the ear.

Aural myiases.—The "screw-worm," the larva of Cochliomyia americana, is the most common cause of aural myiasis in tropical America. The fly deposits its eggs about aural and nasal cavities of those with offensive discharges. The larvae gain entrance to the cavities of the head and develop, causing intense pain and giddiness. Larvae of Sarcophaga, Calliphora and Anthomyia have also been reported from the external auditory meatus. The tympanic membrane may be perforated by them.

# CHAPTER XXVIII

## DIAGNOSIS OF INFECTIONS OF THE TEETH

DENTAL infection is responsible for a number of systemic diseases, and early recognition and eradication of the focus is essential in these cases. Although it is true that many individuals carry demonstrable evidence of dental infection for many years without apparent interference with their health, yet in other cases a slight periapical infection may result in serious systemic disorders. In some of these cases removal of the affected tooth relieves the condition, but in other cases the trouble may persist. It must be remembered that these infections extend into the cancellous bone around the root, and in some cases, particularly those in which the condition has been present for a long time, a residual infection may remain in the bone for years after the extraction. Therefore, a tooth with a demonstrable infection around the apex should be removed promptly if the patient is suffering from any type of focal infection. In a healthy individual conservative dental treatment may be justified, but the potential danger in such an area should be recognized by both dentist and physician. In some cases it is impossible to determine definitely whether or not a devitalized tooth is infected at the root, and it is for the physician to decide on the basis of the patient's general condition whether or not such a tooth should be extracted. In all questionable cases dentist and physician should cooperate closely.

Types of Dental Disease.—Although periapical infection is of chief importance in medical work, other conditions such as extensive pyorrhoea alveolaris, faulty development of the teeth, and caries may also be associated with disturbances elsewhere in the body. Specific infections of the mucous membranes and gums such as Vincent's stomatitis are discussed elsewhere.

Methods of Examination. Clinical Examination.—Each tooth and its contiguous tissues should be examined by means of mouth mirrors, explorers, and dental tape. Absence of teeth, malocclusion, caries, gingival infections, and calcareous deposits should be noted. The detection of devitalized teeth is most important medically, since a chronic, symptomless periapical infection may be present. Such an abscess around a vital tooth is rare but may occur, particularly in cases of extensive pyorrhoea. The

vitality of each tooth may be tested roughly by the application of heat or cold, but preferably with an electric pulp tester. By controlling the amount of current used it is possible to detect teeth with impaired vitality as well as those which are entirely non-vital.

Roentgenological Examination.—This is of value in the detection of uncrupted teeth, tartar, and caries under fillings or in inaccessible areas. Foreign bodies or fragments of a broken root may be found. Occasionally the apices of one or more of the upper first molars and bicuspids (less often the canines) may be seen to penetrate the bone of the floor of the antrum. Infection around such a tooth may cause an intractable maxillary sinusitis. Of particular importance to the physician are the changes associated with periapical infections, and evidences of residual infections following the extractions.

Radiographs should be examined carefully with the naked eye and with a lens against a suitable background. The pulp chamber, the contour of the root, the peridental membrane, and the lamina dura should be examined from the crown to the apex. The crests of the interdental septa, and finally the cancellous bone around the root are examined for evidences of rarefaction, sclerosis, and changes in the bone pattern. Some of these changes are obvious to the naked eye, whereas others are best seen with a lens. It must be remembered that a negative X-ray does not exclude the presence of a periapical infection, either recent or long-standing. In fact extensive necrosis of the jaw may occur without X-ray changes until a sequestrum separates and can be outlined. On the other hand a sclerosed or rarefied area does not invariably mean that living bacteria are still present.

Bacteriological Examination.—Bacteriological studies are of practical importance chiefly in infections of the pulp and periapical tissues. Cultures from the typical chronic periapical abscesses show, in a great majority of the cases, the alpha (viridans) streptococcus. The strains isolated may be subdivided by their fermentative action on carbohydrates into various subtypes, of which the S. fecalis, S. mitis, and S. salivarius are the most common. According to Appleton they do not form any definite serological group. As a rule they may be grown aerobically as well as anaerobically on suitable media. They are relatively avirulent for animals, although Rosenow, Haden and others have produced lesions in animals analogous to the extraoral lesions occurring in the human cases from which the culture was obtained. (See chapter on focal infection.) Occasionally beta streptococci, staphylococci (aureus and albus), other pyogenic cocci, and diphtheroid bacilli may be found alone or associated with the alpha or alpha prime streptococcus. Such organisms are more commonly found in the more acute type of abscess in Appleton's experience.

The presence of active infection may be demonstrated by cultures at the time of extraction. The mouth should be cleansed, and the neighboring tissues painted with iodine or other antiseptic solution. The tooth is then surrounded with sterile cotton rolls to prevent contamination by saliva. Cultures may be made in various ways. If a sac is extracted together with the tooth, cultures may be obtained from the center after searing the outside of the sac. If there is no definite sac material may be obtained from the base of the socket with a sterile swab or curette immediately after the extraction, with care that the instrument does not touch the gingival margins. Contamination is more common, however, by this method. The surface of the extracted tooth may be flamed or otherwise sterilized, and cracked open with sterile instruments. Cultures are then made from the pulp cavity near the apex. Appleton obtains cultures from suspected teeth in situ by puncture through the overlying soft tissues and alveolar plate.

Such a procedure, however, can only be done by a dentist, trained in bacteriology, or by the dentist and bacteriologist in cooperation.

The media used must be enriched with blood or ascitic fluid. It is desirable to inoculate at least one plate in order to determine the purity of the culture obtained, and to get a rough idea of the number of organisms present. Deep tubes of glucose brain agar provide varying degrees of oxygen tension, although many of the strains grow equally well aerobically. When the material is scanty, and the possibility of contamination is slight, a fluid medium such as Rosenow's glucose brain broth can be used, but particular care must be used in the interpretation of the results of such a culture.

In cases of infectious arthritis apparently dependent upon dental infection alone, it is our practice to prepare a vaccine from the culture obtained from the apical region at the extraction. If, after a reasonable time the condition does not improve, and particularly if hypersensitiveness can be demonstrated by an intradermal injection, vaccine treatment may be instituted.

Experience has shown that it is impossible to determine by clinical examination alone whether a pulp chamber which has been exposed is free from infection, and is, therefore, safe to fill. It would be most desirable if bacteriological studies could be made of all such teeth before the root is filled. It is Appleton's opinion that no root canal should be filled if, after a reasonable amount of treatment, smears and cultures continue to show bacteria, unless root resection is done immediately. On the other hand he has found that an abscess occasionally develops in teeth with negative cultures after filling, but that the proportion of these cases is small. The material from the canal can be obtained with relatively little danger of contamination by sterilizing the coronal surface of the tooth and isolating it by a rubber dam before the canal is opened. The taking of the material for such cultures, however, must be left to the dentist. Detailed descriptions of cultural methods may be found in the authoritative book of Appleton, "Bacterial Infection," 1033.

Haden has obtained positive cultures from the apices of devitalized teeth in over 50% of a large and carefully controlled series. Of the cases with negative X-rays 46% were positive, and of those with X-ray changes indicative of infection 63% were positive. Of a control series of vital teeth only 4.8% showed growth.

Caries.—The etiology of this condition is obscure, and its development depends upon a number of factors. It is due primarily to decalcification by the acid formed by bacteria acting upon carbohydrates present around the teeth. Bacteriological studies of carious spots have not shown that any one organism is responsible for the condition and it seems probable that various species can be concerned provided that they possess the ability to grow in such areas and to ferment carbohydrates. However, many other factors are important, such as proper position and occlusion of the teeth to prevent food accumulations, cleanliness, masticatory exercise, etc. In addition metabolic or nutritional disturbances may in some instances cause a disturbance in the calcium-phosphorus ratio and render the teeth more susceptible to caries.

Chronic General Periodontitis. (Pyorrhoea alveolaris).—There is as yet no general agreement as to the primary cause of this condition. Some investigators regard it as a progressive infection starting under the gingival margins, involving the peridental

membrane and cementum with subsequent rarefaction of the alveolar crest. Others consider that the infected pockets are secondary to atrophy of the alveolar margins. This in turn may be the result of various constitutional disturbances. Box distinguishes two types of the disease differing in their etiology. Whatever may initiate the process, however, there is no doubt that it may be aggravated by the presence of calcareous deposits, food accumulations, faulty occlusion, and trauma of various kinds. Mild cases do not ordinarily affect the general health, but when deep pockets are present which do not drain readily, lateral abscesses may form in the alveolar bone along the side of the root. These areas may constitute a focus of infection analogous to the chronic periapical abscess resulting from an infected root canal.

Cultures from the pockets show, as might be expected, a number of species of organisms, and it is impossible to ascribe an etiological relationship to any of them. Alpha streptococci are very frequently present along with other cocci that are able to adapt themselves to anaerobic conditions. The fusiform bacilli and spirochaetes of Vincent are often found in smears together with other mouth spirochaetes. When these are present in large numbers, treatment with spirochaeticidal drugs such as arsenic or bismuth may be a useful adjunct to the usual local treatment. Otherwise bacteriological studies are of little value clinically. Vaccine treatment with one or more of the organisms isolated from a pocket has been tried, but the results are not convincing.

Periapical Infection.—An acute abscess may develop from infection of the root canal through a deep carious area, or from an extension or a flare up of a chronic abscess. In such cases the attendant pain usually drives the patient to a dentist promptly for treatment. If neglected, the abscess may rupture through the bone and periosteum and form a draining sinus either orally or externally. This condition concerns chiefly the dentist, and does not ordinarily result in systemic disease save when a chronic bone infection persist after treatment.

The symptomless chronic infection, which may develop at the apices of devitalized teeth, on the other hand, is a most important focus of infection. In a great majority of these cases the infection enters the bone through an infected root canal. Occasionally an abscess around the apex of one tooth may extend to an adjoining tooth. In rare instances the infection appears to be haematogenous or lymphogenous in origin. In most of the cases these abscesses can be recognized only by the X-ray, and frequently this evidence is inconclusive.

Periapical infection may result in a sclerosis of the bone around the apex of the tooth, or more commonly, in a rarefaction. In general, sclerosis occurs in individuals whose resistance is good, and cases have been observed in which a lowering of the general health has been followed by the development of a rarefaction in the bone beyond the sclerosed area. While the sclerosis appears to be a protective mechanism, cases have been recorded in which relief was obtained only after the sclerosed bone had been removed, and Price has obtained positive cultures from such bone. An area of rarefaction may appear circumscribed in the radiograph. In these cases there is a mass

of inflammatory tissue surrounded by a denser layer of connective tissue, which often adheres to the tooth at the time of extraction. This type of abscess is relatively innocuous, although bacteria and their toxic products may be disseminated through the lymphatics which penetrate the capsule. In other cases the abscess is of the infiltrating type with ill defined edges. These cases are more difficult to detect by the X-ray, and they are in general more likely to result in systemic disease. Furthermore, on extraction some of the infected tissue may be left within the bone even after careful curettage of the socket. This tissue or granuloma is made up chiefly of granulation tissue from which there may be considerable absorption. In some cases a small amount of pus may be present. In individuals with good resistance the bacteria may eventually be killed off, but recent bacteriological studies by Haden and others indicate that the infection may persist here for many years and continue to act as a focus of infection. This observation is of great medical importance, and affords a reasonable explanation of the fact that some cases of arthritis or other systemic disturbance referable to a dental focus of infection do not improve after extraction.

Residual infection may be recognized radiographically in some cases by a persistent area of rarefaction, or by failure of the tooth socket to be replaced by normal bone. Colyer and Heath attach great importance to the presence in the X-ray of multiple small round areas of rarefaction surrounded by a thin ring of sclerosed bone. These are frequently scattered in clusters about an infected tooth, and may be present in the bone at some distance from it. These may persist for months or years after the extraction, and are believed to mean invariably a residual infection. If the infection is overcome, they appear as small sclerotic spots. If resistance is poor, they may spread and coalesce and cause extensive necrosis of the bone. Colyer suggests that these areas in the radiograph are caused by colonies of living bacteria, but it seems more probable that they are minute granulomata formed in response to a diffusion of the bacteria through the cancellous bone.

#### CHAPTER XXIX

# EXAMINATION OF SPUTUM

In the collection of sputum for examination great care must be taken that the material is actually coughed up from the bronchi or lungs. Secretions from the mouth or nasopharynx are worthless for examination. The best time to secure a suitable specimen is in the morning, and the patient should be instructed to save only what is raised from the chest. It is desirable to have the patient clean the mouth thoroughly before obtaining the specimen. Material from the mouth and pharynx can usually be recognized by its appearance, and by the presence of mosaic-like groups of flat epithelial cells. Material from the bronchi or lungs is either frothy mucus or mucopurulent material, and may contain alveolar cells as well as pus cells.

The gross appearance of the sputum is of importance. Large amounts may be expectorated constantly in bronchiectasis, or in tuberculosis with cavity formation. A large amount appearing suddenly suggests rupture of an abscess of the lung, pleura, or liver. A sudden profuse expectoration of frothy, serous material, which may be pinkish, salmon colored, or frankly blood-tinged, indicates an acute pulmonary oedema that demands immediate treatment.

The color may be white, yellowish or greenish, depending in part on the amount of pus present. Bright green sputa occur in resolving pneumonia and in jaundice. The rusty sputum of pneumonia is familiar to everyone. Bright blood suggests tuberculosis, but may occur in bronchiectasis, abscess, carcinoma and other conditions. Bleeding in the mouth or nasopharynx may simulate haemoptysis.

Ordinarily the sputum is odorless, but in bronchiectasis, cavities, abscess and gangrene, and in fusospirochaetosis the odor may be very foul.

Dittrich's plugs are caseous yellowish or grey plugs formed in the bronchi in bronchiectasis or putrid bronchitis. They have a very foul odor when crushed. Occasionally fibrinous casts of the smaller bronchi may be coughed up in pneumonia or in fibrinous bronchitis.

Curschmann's spirals may be seen as whitish curled threads, which, under the low power of the microscope, appear as central threads surrounded by coils of fine fibrils. They occur chiefly in bronchial asthma.

The examination of an unstained preparation is of great value although often neglected. Small purulent or cheesy particles are selected and flattened out in a thin layer under a cover glass. This is examined for elastic tissue, heart failure cells, fungi, amoebae, and ova of animal parasites. The addition of 10% sodium hydrate to the preparation facilitates the examination for elastic tissue and fungi.

Elastic fibres are highly refractile, wavy fibrils of uniform diameter, often split or frayed at the ends. Unless they show an alveolar arrangement we cannot be sure that they have not come from the food. They occur in destructive diseases of the lungs such as advanced tuberculosis, abscess, and gangrene.

Heart failure cells are large mononuclear cells, possibly endothelial leukocytes, filled with brownish or yellowish granules of blood pigment. The nucleus is usually eccentric, but may be obscured by the pigment granules. They occur in chronic passive congestion of the lung, associated most frequently with mitral stenosis, and also following any type of haemorrhage into the lung. Similar cells filled with blackish granules occur in individuals exposed to a dusty atmosphere. Myelin globules also occur in such cells, but they have no special significance.

Charcot-Leyden crystals are frequently present in bronchial asthma, often adhering to the Curschmann's spirals. They are colorless, thin, pointed, hexagonal crystals. They are present in conditions in which eosinophiles occur, and there seems to be some association between them. They are often found in paragonimiasis, and in the pus of amoebic abscesses discharging through the lungs. Other crystals may occur—fatty acid crystals (especially in Dittrich's plugs), haematoidin, cholesterol.

Fungi.—Actinomyces (ray fungus) is found in actinomycosis of the lung. The vellow "sulphur" granules can usually be seen with the naked eye, and under the low power of the microscope appear as finely granular bodies. After crushing under a cover glass and examining with the high power lens, the masses of mycelial threads arranged radially around the edges and ending in characteristic bulbous tips can be seen. Aspergillus fumigatus may rarely cause a pulmonary mycosis resembling tuberculosis, but is also found as a contaminant in old sputum specimens. These may be recognized by their sterigmata carrying chains of spores. Streptothrix infections of the lung occur. These organisms have long, branching filamentous forms with small swollen buds at the ends resembling spores. Blastomyces causes a fatal infection. These are round or oval, doubly contoured, refractile, yeast-like cells which show budding. In cultures hyphae are formed, but in the tissues only budding forms are present. Coccidioides immitis causes a rare and fatal infection called coccidioidal granuloma. They resemble the blastomyces, but do not form buds. Multiplication is by endospores which appear as a cluster of small round bodies inside the cells. Monilia occur as contaminants, but may also cause pulmonary disease. Both yeast forms and mycelial filaments are found in the sputum. Spores are formed at the tips of the mycelia and at the mycelial nodes.

These fungi are discussed in detail in the section on fungi. Their presence in the sputum should be confirmed by repeated examination of fresh specimens before etiological significance is attached to them, since many fungi occur as contaminants. They can also be seen in stained films, but their structures is better brought out in the fresh preparations. Cultures from the sputum can be made on Saboraud's medium for further identification, but it is often difficult to obtain pure cultures.

Stained Smears.—For the study of the cells of the sputum one can use one of the Romanowsky stains, or haematoxylin and cosin. The eosinophiles which are so characteristic of bronchial asthma are well brought out. In sputum from cancer of the lungs clusters of unusual cells may be found. In mild cases of tuberculosis mononuclear cells are commoner than polymorphonuclears, but since secondary infection is common in tuberculosis, this distinction is of little value.

Examination for Tubercle Bacilli.—To make smears for staining, the sputum should be poured into a Petri dish which is placed over a dark background. Several suspicious particles are picked out with forceps or tooth picks and smeared on slides. Select small, opaque, greyish or yellowish cheesy masses, or purulent streaks. Dry and fix with heat. A mark with a grease pencil about ½ inch from the end prevents stain from running over the surface, and gives a convenient surface to hold with the forceps. Sputum should as a routine measure be stained by the Ziehl-Neelson method and by Gram's method. Staining by Spengler's method permits the use of thick films. Other methods of staining are given in the section on staining.

The finding of acid-fast bacilli is good evidence of the presence of tuberculosis, since non-pathogenic acid-fast organisms do not ordinarily occur in the sputum. The demonstration of Much granules is also of some value if contaminating cocci are removed by digestion with one of the concentration methods. Their recognition is difficult unless they are arranged in rows, and there is visible some indication of the body of the bacillus in which the granules lie. Their identification by morphology alone is uncertain, however, unless they are found associated with typical bacilli.

Concentration Methods.—When the number of tubercle bacilli in the sputum is relatively small they may be demonstrated more easily by one of the various concentration methods. Antiformin digests the sputum and dissolves other bacteria. In place of antiformin one may use liquor sodae chlorinatae (Labarraque's solution) double strength, adding 7½% sodium hydrate. Used in the following way, the tubercle bacillus may be demonstrated in smears, and cultures may be made from the sediment with reasonable assurance of obtaining them in pure culture. Twenty cc. of sputum are mixed thoroughly with 65 cc. of sterile water and 15 cc. of antiformin. Allow to stand for from ½ to 2 hours, shaking occasionally, until the mixture is homogeneous. Centrifuge at high speed for ½ hour or longer, and wash the sediment twice with sterile salt solution. Smears are made from part of the sediment, and the remainder may be inoculated on a suitable medium.

If smears only are desired, the antiformin may be somewhat stronger—to cc. of sputum may be mixed with 20 cc. of 50% antiformin. Digest for to to 30 minutes, shaking frequently. Add an equal volume of water and centrifuge. Remove all of the supernatant fluid and drain for 5 minutes on filter paper. In place of the water one may add 4.5 cc. of a mixture of t volume of chloroform and 9 volumes of 95% alcohol. After sedimentation the bacilli are found on top of the chloroform. A little of the untreated sputum may be used to fix the smear to the slide. If the antiformin concentration is too high in the mixture, the staining properties of the tubercle bacilli are injured, and the specific gravity of the mixture is too high to permit sedimentation.

Digestion of the sputum can also be carried out simply by adding to it an equal quantity of 4% sodium hydrate, shaking and incubating at 37°C. for ½ hour or longer.

After digestion is complete, centrifuge at high speed, decant the supernatant fluid, and neutralize the sediment with a few drops of 8% hydrochloric acid. When neutralization is completed, the sediment usually becomes white or opaque. The reaction of the mixture can be tested with litmus paper. A fixative on the slide is not necessary if properly neutralized. Part of the sediment can be used for cultures or guinea pig inoculation.

Spirochaetes and Fusiform Bacilli.—The demonstration of these organisms in the sputum has assumed great importance in recent years. Diagnosis depends upon their demonstration in stained films, or by darkfield illumination. Before obtaining the sputum it is desirable that the mouth and teeth be thoroughly cleansed to avoid as much as possible contamination from this source. The sputum must be examined when perfectly fresh since the spirochaetes disintegrate rapidly on standing. Special spirochaete stains are not necessary, since the organisms stain well with carbol fuchsin 1-5, heated gently over a flame for a few minutes, or with Löffler's methylene blue for 5 to 10 minutes. The characteristic fusiform bacilli stain easily with any method. In fusospirochaetal infections they are constantly present in large numbers. If only a few organisms are found, one should be cautious in their interpretation, since they are frequently present in the mouth. Other organisms, especially (aerobic and anaerobic) streptococci, are commonly found with them, and their pathogenicity alone is questioned. However, their demonstration is of considerable importance since appropriate therapy with arsenic preparations is of great value in these cases.

Pneumococci are best demonstrated by a Gram stain. In sputum from cases of lobar pneumonia they are often present in large numbers, and when surrounded by a definite capsule, their presence may be considered significant. Methods for typing them from the sputum are given in the section on the pneumococcus. However, pneumococci occur often in sputum from conditions other than pneumonia, and are frequently present in the normal mouth.

For the demonstration of other common organisms in stained preparations it is desirable to wash the sputum if possible before making the films, as in making sputum cultures, in order to lessen the number of contaminating bacteria from the mouth. Even in such a preparation the presence of some organism is significant only if it occurs in great preponderance. Such organisms as the streptococcus, pneumococcus, influenza bacillus, staphylococcus, Friedländer bacillus, and N. catarrhalis may be recognized in a Gram stain, or preferably by culture. They are found in a variety of inflammatory conditions in the bronchi and lungs, sometimes as the etiological agent, and at other times as secondary invaders. These organisms, together with various saprophytic bacteria, are often present in tuberculous lesions, especially cavities. Their significance, therefore, can only be interpreted by considering the clinical aspects of the case.

Sputum Cultures.—A tenacious, mucopurulent mass is selected, and placed in a dish of sterile salt solution in which it is gently agitated. This process is repeated in as many changes of salt solution as its consistency permits. The particle is then placed at one side of a blood agar plate, and thoroughly broken up with a platinum loop. The surface of the plate is then streaked over with the loop or a bent platinum wire. If the sputum could not be thoroughly washed or if organisms are numerous, a second plate should be streaked without recharging the wire in order to obtain discrete colonies. In this way a practically pure culture may sometimes be obtained.

Cultures of *H. pertussis* are obtained more readily by holding an open plate of the Bordet-Gengou medium about 4 inches in front of the mouth of a patient with whooping cough during a paroxysm of coughing. The organism may be obtained in a large percentage of cases in the early stages in this way.

For culturing the tubercle bacillus a special technique is necessary. A number of methods have been devised, in which other bacteria are killed and the sputum digested by the addition of alkali or acid. After digestion the mixture is neutralized, and the sediment is inoculated on suitable media. The following methods are recommended.

Petroff's method.—The sputum is thoroughly mixed with about double its volume of 4% sodium hydrate, and placed in the incubator at  $37^{\circ}$ C. for  $\frac{1}{2}$  hour, shaking occasionally. The mixture is then neutralized with 8% hydrochloric acid and centrifuged at high speed for  $\frac{1}{2}$  hour. The sediment is then inoculated on slants of Petroff's medium or Corper's crystal violet potato. The preliminary digestion can be carried out with 6% sulphuric acid or 5% oxalic acid if the mixture is thoroughly stirred. The sediment should then be neutralized with sodium hydrate, or washed with sterile salt solution.

Corper has recently suggested a simple method for culturing tubercle bacilli from the sputum in a little citrated blood or egg yolk. One half to 1 cc. of finely divided sputum (ground up in a mortar if necessary) is placed in each of several test tubes, 6 inches by  $\frac{34}{4}$  inch, stoppered with a paraffined cork. Add to each  $\frac{1}{2}$  cc. of sterile citrated blood or egg yolk and 2 cc. of 6% H<sub>2</sub>SO<sub>4</sub>. Mix and incubate at  $37^{\circ}$ C. for 45 minutes, shaking occasionally. Add slowly enough of a sterile mixture of 1.3% sodium bicarbonate in 3% glycerin to neutralize. The amount necessary is determined by neutralizing separately 2 cc. of the sulphuric acid, using bromthymol blue 0.04% as an indicator and titrating to a deep greenish blue (pH 6.8) or light blue (pH 7.4). Place in the ice box over night, and then pour off the excess of fluid. Stopper tightly and incubate in the dark at least 3 weeks. Then examine at weekly intervals for 12 weeks, shaking the tubes and making smears from a drop of the culture.

Many workers believe that the digestion with acid affects the viability of the tubercle bacillus less than does that with alkali.

The blood-stained, watery sputum of plague pneumonia should be cultured on plates of plain agar and 3% salt agar at the same time. An ordinary smear stained with carbol thionin, however, practically makes a diagnosis. Inoculate a guinea pig cutaneously.

Fungi should be cultured on Sabouraud media as described in the section on fungi.

Animal Inoculation.—The inoculation of a mouse at the root of the tail or intraperitoneally is a useful method for isolating pneumococci from the sputum. Pure cultures can usually be obtained from the heart's blood, and an emulsion of the peritoneal exudate can be used for typing. The details of this procedure are given in the section on the pneumococcus.

For the demonstration of tubercle bacilli contaminating bacteria should be killed by one of the digestion methods. The pig is injected subcutaneously in the groin and watched for from three to six weeks. The characteristic lesions are described in the section on the tubercle bacillus.

Guinea pigs may be infected with plague bacilli by simply rubbing the infected material on the shaven skin.

Rivers (1935) reports the production of characteristic intracellular bodies in the spleens of mice injected with psittacosis sputum either filtered, or unfiltered if no pneumococci or streptococci are present. Andrewes et al. have produced pneumonia in mice by intra-nasal inoculation with filtered extracts of influenza virus, and it seems probable that this may become of use in the diagnosis of this disease.

Albumin test.—About 10 cc. of fresh sputum as free as possible from saliva is mixed with an equal quantity of water and 2 cc. of a 3% solution of acetic acid to remove mucin. After filtering, the filtrate is tested for albumin as in urine. If more than a trace is present it can be estimated quantitatively by Esbach's method. Normally only a trace is present. It is increased in amount in tuberculosis and pneumonia.

Vaccines.—The value of vaccine therapy in the acute respiratory infections is dubious, but in chronic infections, especially in chronic bronchitis associated with asthma, autogenous vaccines may be of benefit.

The organisms most frequently obtained from the sputum in these cases are the streptococcus (viridans or hemolyticus), the pneumococcus (rarely one of the fixed types), and the influenza bacillus. Other varieties mentioned above are less commonly present. In preparing a vaccine, smears and cultures must be examined with great care to determine the predominant type of organisms. If more than one pathogenic species is present in the culture in large numbers, a mixed vaccine can be used. Vaccines made from contaminating bacteria from the mouth, or from saprophytes, cannot be expected to have any specific effect on the infection.

Animal Parasites.—Amoebae from a liver abscess rupturing into the lung may be found. Very important pulmonary infections are those with *Paragonimus ringeri*. This is recognized by the presence of operculated eggs in the sputum.

A fluke, F. gigantea, was once found in sputum.

Hydatid cysts, either of the lung, or of the liver rupturing into the lung, may be recognized by the presence of *Echinococcus* hooklets. The material is bile-stained if from the liver. Dutcher has reported filarial embryos from sputum.

Metastrongylus apri has been reported once from the lungs and embryos might be found in the sputum. As the larval forms of hookworms, Strongyloides and Ascaris go by way of the lungs and trachea these might be found in sputum at time of migration.

# CHAPTER XXX

# EXAMINATION OF PUS

Pus may be collected for examination either (1) with a platinum loop, (2) with a sterile swab, (3) with a bacteriological pipette or (4) with a hypodermic syringe.

It is always well to make a smear and stain it by Gram's method at the same time that cultures are made. The Gram stain gives information as to the abundance of organisms in the pus and as to the probable findings in the culture. Pneumococci and streptococci are differentiated from the staphylococci in this way without the necessity of extended cultural methods.

The hypodermic syringe is very useful in puncturing buboes, etc., especially in plague. A small pledget of cotton on a toothpick dipped into pure carbolic acid and touched to a spot over the bubo, the escharotic action being arrested with alcohol after about thirty seconds, makes a sterile anaesthetic spot at which to introduce the needle of the syringe.

A bacteriological pipette is very useful when pus is to be sent to a laboratory; the tip can be sealed in a flame and the cotton plug at the other end insures the noncontamination of the contents. The material may be drawn up either with the mouth or with a rubber bulb.

Smears from material examined for gonococci may show Gram-negative diplococci which, however, may not have the typical morphology of the *Gonococcus*. They are furthermore often extracellular.

The *N. catarrhalis* has been reported from urethral smears though very rarely. Diphtheroid organisms are not uncommon. Gram-positive cocci are rather common in smears from discharges of chronic gonorrhoea.

War wounds.—In a study of the aerobic bacterial flora of war wounds Lawrence found that more than 80% of the discharges from such wounds showed streptococci, which especially flourished in deep pockets, staphylococci replacing them in shallow wounds. Gram-negative bacilli were present in 95% of smears. Of these, E. coli was present in 50% of cases. The combination of aerobes and anaerobes in a wound makes conditions more favorable for the anaerobes. Wounds contaminated with fusiform bacilli do badly.

The pus from wounds infected with anaerobes is usually very foul. The most important anaerobe in the discharge from gas gangrene wounds is Cl. welchii.

The pus from the necrotic center of climatic bubo is sterile.

It is remarkable how frequently we get pure cultures from abscess material. In purulent material from abdominal abscess we are apt to obtain mixed cultures, especially the colon bacillus and pyocyaneus bacilli, in addition to ordinary pus organisms.

In examining blood-serum or blood-agar slants inoculated with purulent material, always examine the water of condensation for streptococci. When it is a question between streptococci and pneumococci, it is well to inoculate a mouse; finding the capsulated pneumococci at the autopsy makes the diagnosis.

When the plague buboes begin to soften, the plague bacilli may be replaced by ordinary pus organisms.

Animal inoculation also is often necessary in plague and glanders, and sometimes in anthrax. When tetanus is suspected, it should be searched for as described under Tetanus. Tuberculosis should be identified by inoculating a guinea pig, as well as by acid-fast staining and culture, if there is any doubt as to the nature of the material.

The black or yellow granules of madura foot, as well as those of actinomycosis, should be examined as recommended in the section on fungi.

Amoebae, coccidia, and larval echinococci may be found in purulent material, as may also various other animal parasites, as fly larvae, sarcopsyllae, etc.

The pus from an amoebic abscess of the liver is as a rule sterile when cultured, and the examination at the time of operation or exploration frequently shows an absence of amoebae as well as of bacteria; but two or three days later amoebae may be found in the pus draining from the abscess cavity.

Flukes, round worms and whip-worms may as a result of their wandering from the intestinal lumen cause abscesses.

Serious ulcerations may follow infection with the Guinea-worm.

Abscesses often occur about encysted filarial worms.

#### CHAPTER XXXI

# SKIN INFECTIONS

Cultures should be made, as a rule, in the bacteriological examination of lesions of the skin. The surface should first be washed with soap and water in order to eliminate chance organisms from dust or other extraneous sources. Scrapings are then made with a sterile dull scalpel, and the material obtained is emulsified in a drop of sterile water in a Petri dish. A tube of melted agar at 42°C, may be poured over the drop and mixed, or the material may be spread over the surface of an agar plate. About 80% of the colonies developing will be Staphylococci and the greater proportion will be white colonies.

Occasionally S. aureus or S. citreus may be isolated. S. aureus is the organism usually isolated from furuncles, circumscribed abscesses and carbuncles. Streptococci and colon bacilli are rarely found. Streptococci are the organisms to be expected in phlegmonous infections. Cold abscesses, which are frequently due to tuberculous infection, as a rule are sterile. Acne pustules may show staphylococci, the acne bacillus, or both.

Corynebacterium acnes (Bacillus acnes) is a short, usually pleomorphic bacillus about 1.5 by 0.5  $\mu$  in size, which often shows a beaded appearance when stained by Gram's method. It is Gram-positive. It grows readily on glucose agar when cultivated anaerobically, and also aerobically on acid media containing blood serum. Colonies appear in 4 to 5 days.

The "bottle bacillus" (Malassezia ovalis), which has the morphology of a yeast, is regarded as the cause of dry pityriasis capitis. It may also be found in the comedones of children.

In the tropics an organism which is probably a virulent strain of Staphylococcus aureus at times produces lesions similar to impetigo, at other times pemphigoid eruptions or wide-spreading erysipelatous conditions. It has been described under the name of Diplococcus pemphigi contagiosi.

Staphylococcus epidermidis, the common cause of stitch abscesses, is considered by Sabouraud to be the cause of eczema seborrhoicum.

In scrapings from the skin of lepromata the acid-fast bacilli are found in the greatest profusion. In tuberculosis of the skin the tubercle bacilli are exceedingly scarce. Inoculation of a guinea pig usually gives positive results with the tubercle bacillus. The leprosy bacillus is non-pathogenic for laboratory animals.

Anthrax and glanders cause skin lesions which can be surely diagnosed only culturally or by animal inoculation.

Plague bacilli may be isolated from the primary vesicles appearing at the site of the flea bite.

Tropical phagedaena is thought by some to be due to a sort of diphtheroid organism. The organisms of Vincent's angina may cause tropical ulcer.

Granuloma venereum.—In scrapings from the ulcerations on the external genitals and adjacent skin Donovan reported finding macrophages containing numerous small bacilli, Klebsiella granulomatis. These are Gram-negative, encapsulated, oval, diplococoid organisms 1.5 by  $2\mu$ . They can be cultivated readily on Sabouraud's and other media, and in cultures they resemble the Friedländer bacillus. Although these organisms are almost constantly present, it is possible that they are secondary invaders rather than the primary cause of the disease. Although this venereal disease (not to be confused with lymphogranuloma inguinale) is largely limited to colored races in the tropics, especially in the West Indies, cases have been reported in negroes in the southern United States.

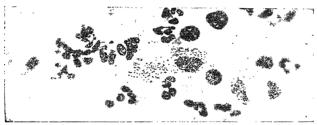


FIG. 171.—Klebsiella granulomatis. In a scraping from an ulcer from a case of granuloma venereum. Tropical Institute, Leiden. (After Flu from Ruge, Muhlans and zur Verth.)

The skin diseases due to fungi, which are widely prevalent, are discussed in Chap. X. Animal Parasites.—Certain skin diseases, as Oriental sore, are protozoal in origin. The cutaneous lesions of uta or espundia are now known to be caused by a *Leishmania* as well as is Oriental sore. These affections in the Central and South American countries are now known as American leishmaniases.

Of the skin eruptions caused by animal parasites, ground itch (dew itch, foot itch) is the most important. This is a dermatitis caused by the irritation set up by the hookworm larvae penetrating the skin of the foot and leg.

According to Lemaire, ten species of nematode larvae, other than those parasitic in man, may penetrate the skin, setting up a dermatitis. Not being adapted to man they die out without infecting other tissues. One type of such dermatitis is the "creeping eruption" caused by larvae of the dog hook worm. A similar eruption is caused by larvae of the bot fly of horses (Gastrophilus).

Filarial infections are also important, especially the ulcers of the Guinea-worm, Calabar swellings of *Loa loa*, the cystic tumors of *Onchocerca volvulus* and the varicose groin glands and elephantiasis of *W. bancrofti*.

Enterobius (Oxyuris) may cause a severe irritation about the region of the groin and inner surfaces of the thigh, and especially about the vulvar region of female children.

The larvae of *Gnathostoma spinigerum*, a nematode with two lip-like structures and spine-like appendages covering its anterior one-third, have been found in tumefactions of the skin.

Plerocercoid larvae of Diphyllobothriidae have been found in the subcutaneous tissues.

Leeches, as H. zeylanica, may cause serious ulceration.

The Tunga penetrans (Sarcopsylla penetrans) or jigger (sand flea) is an important agent in ulcerations about the foot.

Certain acarines cause skin lesions, as do also the larvae of certain flies (see Chap. XXII).

The itch mite (Sarcoptes scabiei) is an important animal parasite of the skin.

The various lice, fleas and bedbugs are well recognized causes of skin irritation.

## CHAPTER XXXII

# EXAMINATION OF THE CEREBROSPINAL FLUID AND OF FLUID FROM THE SEROUS CAVITIES. CYTODIAGNOSIS

## THE CEREBROSPINAL FLUID

THE average normal volume of cerebrospinal fluid is 100 to 150 cc. The normal cerebrospinal fluid pressure with the patient on his side in the recumbent position is 5 to 12 mm. of mercury or 70 to 160 mm. of water. The difference in the pressure by the two methods is due to the difference in the specific gravity of the fluids used. It is sufficiently accurate for clinical purposes to multiply the readings of a mercury manometer by 13.5 to convert to water manometer readings, or to divide by 13.5 to convert water manometer readings to mercury readings. Cerebrospinal fluid pressure above 15 mm. of mercury or 200 mm. of water is considered increased. The increase in cerebrospinal fluid pressure may be due to any one of four factors: (1) Increased rate of secretion. (2) Decreased rate of absorption. (3) Loss of continuity of the subarachnoid space or obstruction of the connecting foramina. (4) Increase in brain volume. The rate of secretion is increased in all inflammatory conditions, and unless the rate of absorption keeps pace with the rate of secretion we have an increased volume of cerebrospinal fluid and an increase in pressure. In acute inflammatory conditions the rate of absorption lags behind the rate of secretion, thus producing an increased pressure. If there is any block or obstruction of the subarachnoid space, the cerebrospinal fluid pressure rises cephalad to the block. Increase in cerebrospinal fluid pressure may occur with brain tumors or abscesses in certain locations in the brain.

## Character of the Normal Cerebrospinal Fluid

Normal cerebrospinal fluid is crystal clear and colorless. There is no pellicle, clot or sediment of any kind. The specific gravity ranges from 1.001 to 1.008. The pH ranges from 7.4 to 7.6. The alkali reserve or carbon dioxide combining power parallels that of the blood plasma and ranges from 50 to 65 vol. % at 0°C. and 760 mm. pressure.

Cytology.—Using fresh fluid the average normal number of cells in individuals over five years of age is 1 to 6 per cmm. A slight increase may be allowed for children under five years of age. Counts ranging from 6 to 10 are to be regarded as suspicious, and

counts above 10 are to be considered pathological. The only cell found in normal cerebrospinal fluid is the small lymphocyte; rarely do we find a single polymorphonuclear leukocyte. Normal cerebrospinal fluid contains no red cells and no fibrinogen, therefore the fluid will not clot.

Chemistry.—The cerebrospinal fluid sugar is 20 to 30 mg. per cent lower than the sugar content of the blood. There appears to be a definite relationship between the cerebrospinal fluid sugar and the blood sugar, with a considerable lag in the rise of the cerebrospinal fluid sugar. The average findings are 50 to 80 mg. per 100 cc. of spinal fluid with a blood sugar ranging from 80 to 120 mg. per 100 cc. of whole blood. Any spinal fluid sugar found out of this average range should be again investigated and a blood sugar determination made at the same time.

The chloride content of the cerebrospinal fluid, expressed as sodium chloride, averages 720 to 750 mg. per 100 cc.

Protein.—The total protein of normal cerebrospinal fluid varies from 15 to 40 mg. per 100 cc. Ventricular fluid contains very little protein; it may be as low as 5 mg. per 100 cc. The albumin averages 20 mg. per 100 cc.; globulin, 6 mg. The non-protein nitrogen content parallels that of the blood very closely, the average finding being 15 to 35 mg. per 100 cc.; urea nitrogen, 10 to 15 mg.; uric acid, 1.0 to 2.0 mg.; creatinine, 1.0 to 2.0 mg.

## Pathological Changes in the Cerebrospinal Fluid

Pleocytosis.—Strictly speaking, pleocytosis means an increase in the lymphocytes in the cerebrospinal fluid. An increase in cells in the cerebrospinal fluid is usually an indication of an inflammatory condition. The character of the cells, whether lymphocytes or polymorphonuclear leukocytes, is of diagnostic importance. An inflammation due to cocci is attended by an increase in polymorphonuclear leukocytes, whereas viruses, treponemata, and tubercle bacilli usually produce a lymphocytic response. Irritation by chemicals, inflammation of neighboring structures (mastoid, sinuses, etc.) and the introduction of foreign protein into the subarachnoid space will also produce a mononuclear cellular increase.

An increase in the cell count of the cerebrospinal fluid will produce a change in the transparency through stages of a ground glass appearance to frank turbidity, depending upon the number and type of cell present. As a general rule it takes a larger number of lymphocytes (500 or more) than polymorphonuclear leukocytes (200 or more) to produce turbidity. The cerebrospinal fluid is usually recorded as clear, opalescent, turbid, or purulent. Qualifying adverbs such as faintly, distinctly or markedly may be used to record finer shades of transparency than those given above, but are usually unnecessary.

Erythrochromaemia.—This is a term used to describe a red or reddish fluid due to blood or haemoglobin. If the red color is due to fresh blood, when the fluid is centrifuged the supernatant fluid will be clear and the red cells packed in the bottom of the tube. In old haemorrhage the supernatant fluid is yellow.

Xanthochromia.—This term is used to designate a yellowish color due to altered haemoglobin in the fluid, with no red blood cells. The cause usually given for this color is that the spinal veins have been compressed and exudation of protein, corpuscles and

plasma has taken place. The haemoglobin disintegrates, giving rise to a yellow fluid containing a marked increase in protein, and the fluid coagulates spontaneously (Froin's syndrome). Xanthochromia is not diagnostic of any specific lesion, as tumors of the cord, tuberculous meningitis, block from meningitis, gumma, extensive adhesions, or fracture of the spine may obliterate the continuity of the subarachnoid space and produce xanthochromia below the lesion.

The fluid may have a green or grey color in suppurative cases; and in long-continued cases of jaundice there may be sufficient bilirubin present to give a yellow color.

Coagulation.—The normal cerebrospinal fluid contains no fibrinogen, therefore it does not coagulate. Any infectious condition which allows cells and fibrinogen to pass into the spinal fluid permits coagulation to take place when exposed to the atmosphere. A fine, fragile, cobweb-like pellicle suggests tuberculous meningitis; a clump-like coagulum suggests a suppurative condition; a precipitate-like coagulum is often seen in syphilis.

Albumin and Globulin.—The albumin is increased in practically all pathological conditions. Increase in globulin is taken to indicate disease of the central nervous system, particularly syphilis. Cerebrospinal fluid containing an increase in globulin without an increase in the cellular content (Nonne's syndrome) should make one suspect cord tumor.

Sugar.—Infections due to cocci produce a low cerebrospinal fluid sugar, the severity of the infection governing the decrease in sugar content. A more gradual fall in sugar occurs in tuberculous meningitis. An increase in sugar, above 160 mg., indicates definite need to investigate the patient for diabetes mellitus.

**Chlorides.**—The estimation of chlorides (expressed as sodium chloride) is important from a diagnostic standpoint. In tuberculous meningitis the chlorides fall rapidly to less than 600 mg. A lesser reduction (680 to 630) occurs in acute purulent meningitis.

### Cerebrospinal Fluid Changes in Various Diseases

Acute Anterior Poliomyelitis.—Early in this disease the cerebrospinal fluid will be clear with a moderate increase in cells (20 to 30, of which 90% or more are lymphocytes). After several hours a second puncture may yield fluid containing 50% or more of polymorphonuclear leukocytes, while a subsequent puncture reveals a shift to lymphocytes again. This cellular shift from polymorphonuclear leukocytes to lymphocytes, when found, definitely suggests anterior poliomyelitis. There are no other abnormal findings.

Acute Benign Lymphocytic Choriomeningitis.—In this disease the cerebrospinal fluid is under increased pressure with an increase in lymphocytes. There are no other pathological findings.

Alcoholism.—In acute alcoholism the cerebrospinal fluid will be found under increased pressure and alcohol will be found in the fluid. (See Determination of Alcohol in Blood, p. 679.)

Diabetes Mellitus.—In coma from diabetes the cerebrospinal fluid shows an increase in sugar, no increase in cells, and a pressure which is lower than normal.

Epidemic Cerebrospinal Meningitis.—In this condition the pressure is increased; the fluid is cloudy; there is an increase in cells, 85% of which are polymorphonuclear leukocytes; and Gram-negative cocci (*Neisseria intracellularis*) are present.

Epidemic Encephalitis.—During the acute stage there is often a moderate increase in globulin and in cells which are all lymphocytes. In chronic cases with the Parkinsonian syndrome the fluid is normal.

Lead Poisoning.—The cerebrospinal fluid shows an increase in pressure and an increase in lymphocytes, and the dithizone test may show definite evidence of lead. There are signs of hypertensive encephalopathy.

Multiple Sclerosis.—During the active progressive periods of the disease the globulin is markedly increased, but the cells as a rule are not notably increased. There may be a paretic type of gold chloride reaction, but a negative Wassermann reaction.

Subarachnoid Haemorrhage.—The fluid is under increased pressure, is grossly bloody, and after the cells have been removed by centrifugalization shows a distinctly yellowish color.

Syphilis.—The cerebrospinal fluid is under increased pressure; the cell count is increased (usually 25 to 100 lymphocytes); the globulin is increased; the Kahn and Wassermann reactions are positive. A colloidal gold curve in the first zone indicates paresis; a second zone curve is found in other types of involvement of the central nervous system (tabes, syphilitic meningitis, etc.).

Tuberculous Meningitis.—This disease causes an increase in pressure, an increase in lymphocytes, and a decrease in sodium chloride and usually in sugar content. This finding is practically pathognomonic.

#### Lumbar Puncture

Select an appropriate intervertebral space, either the third or fourth lumbar. Anaesthetize the overlying skin, using an especially sharp, small needle. Select a non-breakable, sterilized, sharp, short-beveled spinal puncture needle with an accurately fitting stylet. Mark the spot in the anaesthetized area you desire to enter with the thumb nail of the gloved left hand, then gently and slowly but firmly pass the needle in the midline and straight in. After the skin has been pierced, realign your needle and enter the fixed supraspinous ligament. This is probably the most important step in a successful puncture, as the direction of the needle is very difficult to alter after entering this structure. The needle will pass with ease and practically no resistance the loose areolar tissue which is next encountered. The second point of resistance will be the ligamentum flavum. This ligament produces sense of resistance and the stylet may be withdrawn at this time. If no fluid is obtained reinsert the stylet and again push the needle a millimeter or two further where one again meets resistance on puncturing the dura. As the needle punctures this inelastic membrane a definite sense of "give way" and palpable "click" is felt. Remove stylet and if the lumen of the needle is in the subarachnoid space, cerebrospinal fluid will be obtained. If no fluid is obtained rotate the needle a half turn, again remove stylet and observe for flow of fluid.

This procedure of withdrawing stylet may be performed from time to time during passage of the needle if one is not sure that the needle point is in the canal. Before withdrawing the stylet completely have the manometer ready and always take the spinal fluid pressure. It is best to rotate the needle and again take pressure as the lumen may be partly blocked or only partly in the canal. This is especially important in all

acutely ill patients and those suspected of increased intracranial pressure. In the case of "dry tap" the needle may be rotated, slightly withdrawn, or pushed in a little as no flow usually means that the needle point is not in the subarachnoid space.

If one inserts the needle too far the venous plexus on the anterior wall may be cut and bloody fluid obtained. If the needle is withdrawn a very short distance this bleeding will usually cease after a few cubic centimeters of fluid have been obtained. The second portion of fluid, if clear, may be sent to the laboratory for examination.

A "bloody tap" is usually due to trauma to a vessel. As a rule the first portion of fluid removed contains more blood than the last. A bloody fluid is practically useless except for a Wassermann reaction and for cultures. If the fluid is only slightly blood-tinged, however, it may be possible to demonstrate an increase in the cell count by making total counts of both red and white cells, and subtracting from the latter the number of leukocytes estimated to have been introduced with the blood (1 for each 750 red cells). A second diagnostic puncture as a rule should be postponed for ten days, until the meningeal reaction to the puncture has subsided.

A fluid uniformly bloody in all fractions is usually due to a subarachnoid haemorrhage, or to an intracerebral haemorrhage which has ruptured into the ventricles.

Caution.—Removal of spinal fluid is dangerous in patients with increased intracranial pressure due to brain tumor. An ophthalmoscopic examination should be made as a routine before every lumbar puncture. Any evidence of swelling of the optic discs calls for an exhaustive neurological examination, and if a puncture is done, the fluid should be removed very slowly and the procedure stopped at the first indication of respiratory disturbance. Lowering a pathologically high pressure is beneficial (at least temporarily) in meningitis and in most other conditions.

The patient should be kept prone in bed for 36 hours after the puncture to lessen the tendency to leakage of cerebrospinal fluid into the tissues through the puncture wound in the meninges. If this happens an incapacitating headache will occur as soon as the patient sits up.

#### Cisterna Puncture

Position of the Patient.—The patient should be recumbent in bed on his right side, with the head supported by a pillow of correct thickness to secure alignment of the cervical with the thoracic vertebrae. The head is flexed as much as possible without producing pain or discomfort.

Operative Procedure.—Except for children no anaesthetic is ordinarily needed. Use a small non-breakable cisternal puncture needle. The thumb of the left hand is placed on the superior occipital protuberance and is caused to slide gently but firmly down the back of the neck until it encounters the bony prominence of the spine of the axis. This space is then marked with iodine, and the associated anatomical structures which guide in reaching the cisterna are carefully explored and visually brought into relation with each other for use in securing correct alignment and direction of the puncture needle as follows:

- The cervical vertebrae are placed in line with the external occipital protuberance and thoracic vertebrae.
  - 2. The head is flexed as much as possible without producing pain or discomfort.
- 3. The external auditory canal is definitely marked with a tightly rolled pencil of cotton.

- 4. The chin, nose and sternum are kept in line by an assistant.
- 5. The glabella, external auditory canal, and needle are now brought into one continuous line.

The needle is thrust through the skin in the midline of the neck over the axis and its full length kept parallel with the external auditory canal. Keeping the needle in midline, proceed cautiously forward and upward with the point of the needle toward the glabella. Proceeding thus, with the landmarks kept correctly aligned, one passes through the anatomical structures without meeting bony resistance. After penetrating to a depth of about 3 cm. one must proceed further with due caution until, as Ayer so aptly describes, he "receives the same sense of 'give way' as experienced in going through the lumbar dura." If in doubt, the stylet should be removed from time to time and the needle observed for flow of fluid. Never insert the needle more than 6 cm. in adults and never more than 3.5 cm. in infants. The cisterna is usually reached in adults at a depth of from 4 to 5 cm., and in infants from 2 to 3.5 cm. and about 1 to 1.5 cm. at the lower level. It is needless to say that one should secure practice on the cadaver.

Diagnostic indications for cisterna puncture only. --

- 1. Suspected subarachnoid block.
- 2. Localized spinal block by using combined cisternal and lumbar puncture.
- 3. Localizing lesions by injection of air or opaque material for roentgenograms.
- 4. Treatment of patients with specific serum, who may be losing ground under injections by lumbar puncture.
  - 5. To relieve headache in block.

#### Osmotic Tap

"Osmotic tap," whether due to loss of fluid by vomiting, diarrhoea, hyperglycaemia or induced by intravenous injection of hypertonic glucose or saline solution, will produce marked lowering of the cerebrospinal fluid pressure. Induced osmotic tap is often spoken of as therapeutic decompression of the brain. For this purpose the use of 100 to 200 cc. of 50% dextrose solution intravenously has been found to be safe and efficient. The second best is 20% sodium chloride solution. The vein above the point of injection of these solutions must not be compressed, as each, in the percentage strength given, is a sclerosing agent.

The clinical application of this procedure is based upon the fact that following the intravenous injection of a hypertonic saline solution, e.g., 20% sodium chloride, the cerebrospinal fluid pressure, after a sharp but very brief rise, falls profoundly for a period of 3 to 5 hours, and the former pressure is not again reached until some 7 or 8 hours later. The venous pressure at first also shows a sharp, sudden, temporary rise, to return to its previous level. The arterial pressure is not notably disturbed.

#### Avala's Ouotient

The quotient is obtained by multiplying the number of cc. of fluid withdrawn (Q) by the final pressure reading (F) and dividing the product by the initial pressure reading (I).

Quotient = 
$$\frac{Q \times F}{}$$

The quotient is a measure of the rate of fall in the cerebrospinal fluid pressure in conjunction with the amount of fluid withdrawn. Determination is indicated in subarachnoid block, meningitis, hydrocephalus or suspected tumor.

The normal quotient is 5.5 to 6.5. Quotients below 5 indicate a small reservoir and speak for tumor mass or block; and quotients over 7.5, for meningitis or hydrocephalus, indicating a large reservoir or increased flow of spinal fluid. This test is not diagnostic but may be helpful in suspected tumor or block.

#### Queckenstedt's Test

This is a valuable test and is indicated in suspected subarachnoid block from any cause and in lateral sinus thrombosis. This test is based upon the fact that in the normal individual the cerebrospinal fluid pressure is rapidly increased if both internal jugular veins are compressed, and is applied as follows:

With a needle in the lumbar subarachnoid space and manometer attached, an assistant compresses both jugulars, and the rapidity of rise in pressure is observed. Failure to obtain a prompt and marked rise in pressure on compression of both jugular veins indicates a subarachnoid block. A similar rise in pressure obtained by compression of one jugular vein only indicates obstruction (usually thrombosis) of the lateral sinus on the opposite side.

## Technique of Examinations of the Cerebrospinal Fluid

Cell Count.—The cell count should be made as soon as possible after the fluid has been obtained. If the fluid is cloudy, add a trace of oxalate to a portion to prevent clotting. The fluid is shaken up, and a drop may be mounted on a haemocytometer chamber. It is preferable to use a staining solution such as:

Crystal violet, o. I Gm.; glacial acetic acid, I cc.; water to 50 cc.; one drop of phenol. Draw staining solution to the mark I in a leukocyte diluting pipet and fill with spinal fluid to the mark II. Shake, mount a drop on a counting chamber, and count the cells as in making a leukocyte count. Multiply by 1% to correct for the dilution.

Differential Count.—A portion of the fluid is centrifugalized, the fluid decanted and thin films made from the sediment. These may be stained by Wright's stain, or they may be fixed with absolute methyl alcohol and stained with aqueous methylene blue. It is necessary only to differentiate polymorphonuclear leukocytes, lymphocytes and large mononuclear endothelial cells. This can be done fairly well in the counting chamber.

Globulin.—An increase in globulin may be demonstrated by:

- I. Ross-Jones test (Nonne-Apelt reaction, phase I).—Over I cc. of saturated ammonium sulphate solution carefully layer 0.5 cc. of clear spinal fluid. An increase in globulin is indicated by the appearance of a thin white "ring" within a few seconds. (A ring may appear after 5 minutes or more with a normal fluid.)
- 2. Pandy's test.—To one cc. of a clear saturated solution of phenol in water (about a 10% solution) add a drop of spinal fluid. An increase in globulin is indicated by the appearance of a bluish-white cloud. A normal fluid may show a faint turbidity. The density of the cloud measures roughly (+1 to +4) the degree of increase in globulin.

Either test will give a positive reaction with a normal spinal fluid which is demonstrably contaminated with blood.

Total Protein.—A quantitative estimation of the total protein in the spinal fluid is important, both for diagnosis and as an aid in estimating the effect of treatment in cases of syphilis.

Method of Denis and Ayer.—Into a test tube of about 4 cc. capacity, 0.6 cc. of spinal fluid is measured. To this is added 0.4 cc. of distilled water and 1 cc. of a 5 per cent solution of sulphosalicylic acid. The contents of the tube is then mixed by inversion (but not by violent shaking) and after being allowed to stand for five minutes the suspension is read by means of a suitable colorimeter against a standard protein suspension prepared at the same time as the unknown. Before reading the standard against the unknown, the standard solution should be placed in both cups and several readings made. In fluids of extremely high protein content, such as may be encountered in cases of spinal cord compression, meningitis, etc., it is sometimes necessary to make a preliminary dilution with water as even 0.1 cc. of such fluids may contain too much protein to read against the standard.

Preparation of standard for total protein determination (Ayer and Foster).—Twenty cc. of fresh normal human blood serum are diluted to 200 cc. with 15% solution of sodium chloride in a volumetric flask and filtered. This filtrate is the concentrated standard.

The total nitrogen of this filtrate is determined by macro-Kjeldahl on 40 cc. The non-protein nitrogen is determined on the original undiluted serum by the micro-Kjeldahl method of Folin and this figure divided by ten is subtracted from the total nitrogen to obtain protein nitrogen. Protein nitrogen multiplied by 6.25 gives the protein content of the concentrated standard.

This concentrated standard is diluted with distilled water to make the dilute standard containing 30 mgs. protein per 100 cc.

These standards are preserved with a few crystals of thymol and kept on ice except when in use. In this way we have kept the concentrated standards for more than six and the dilute standards more than twelve months without appreciable change in protein content.

The standards prepared for estimation of albumin in the urine may also be used (see p. 716).

Colorimetric Determination of Total Protein and Globulin.—(Method of Philip B. Matz, based on the procedures of Wu and of Hewitt.)

The principle of the method depends upon the development of a yellow color from the interaction of Folin's phenol reagent with the tyrosin present in the protein, and comparison of this color with that given by a standard tyrosin solution. Since the tyrosin content of a given protein is constant, the amount of the latter can readily be calculated.

Reagents. Standard tyrosin solution.—Dissolve 50 mg. tyrosin (Pfanstiel) in 250 cc. N/10 HCl. This keeps many months.

Phenol reagent.—In a large Erlenmeyer flask put 100 Gm. of sodium tungstate, 20 Gm. phosphomolybdic acid, 50 cc. of 85% phosphoric acid, 100 cc. of HCl and 750 cc. of water. Put a funnel in the neck of the flask and boil gently for two hours. Cool, dilute to 1 liter and filter. The solution should have a bright canary-yellow color.

Saturated ammonium sulphate solution.

10% sodium tungstate solution.

23 N sulphuric acid solution.

1% sodium hydroxide solution.

20% sodium carbonate solution.

A. Globulin determination.—(1) Put 5 cc. of spinal fluid in a centrifuge tube, add 5 cc. of saturated ammonium sulphate solution, mix, and put in water bath at 37°C. for 30 minutes. (2) Centrifugalize at high speed for 10 minutes, decant fluid, drain thoroughly and remove any remaining drops of fluid with filter paper. (3) Add 1 cc. of 10% sodium tungstate solution, and shake gently until the precipitate is dissolved. (4) Remove solution carefully with capillary pipet to a clean centrifuge tube, add 4 cc. of water and 1 cc. of 2% N sulphuric acid. (5) Centrifugalize and discard the fluid. (6) Dissolve the precipitate in 2 or 3 drops of 1% NaOH, shaking gently. Add 2 cc. of water and 0.1 cc. of phenol reagent. (7) Add 1 cc. of 20% sodium carbonate solution and water to give a volume of 10 cc. (8) At once prepare standard solutions; in a 25 cc. glass-stoppered cylinder put 1.0 cc. of stock tyrosin solution, and in a second put 0.5 cc. (9) To each add 0.25 cc. phenol reagent and water to 20 cc.; then 2.5 cc. of 20% sodium carbonate solution and water to 25 cc. Set all aside for 15 minutes, and then compare in the colorimeter, using the standard that most nearly matches the unknown.

Calculation .-

 $\begin{aligned} \text{mg. tyrosin in globulin} &= \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{concentration of tyrosin in} \\ &\quad \text{standard} \times \frac{\text{Volume of unknown}}{\text{Volume of standard}} \times \text{Volume of spinal fluid} \end{aligned}$ 

The concentration of tyrosin is either 0.1 or 0.2 mg. The volume of the unknown is 10 cc.; of the standard, 25 cc. The volume of the spinal fluid examined is 5 cc. To get the weight of globulin, this figure is multiplied by 25.2, the equivalent of 1 mg. of tyrosin.

B. Total protein.—(1) Put 2 cc. of spinal fluid in a test tube, add 1 cc. of  $\frac{2}{3}$  N HCl and 1 cc. of 10% sodium tungstate solution, and mix. (3) After 30 minutes centrifugalize, decant the fluid, and dissolve the precipitate in 3 drops of 1% NaOH. Then proceed as in steps (6) to (9) above.

To get the tyrosin in albumin, subtract the figure for globulin from that for total protein. To get the weight of albumin multiply this difference by the factor 27.5.

The chief technical difficulty is that the solution is apt to become turbid if ammonium sulphate is carried over in steps (2) to (4).

In pathological conditions the total protein is increased, the globulin much more than the albumin, so that the A/G ratio is diminished and may be actually reversed. Aside from purulent fluids the most marked changes are seen in general paresis and in some cases of multiple sclerosis.

## COLLOIDAL GOLD TEST (LANGE)

The principle of the test depends upon the facts that, if the conditions and the quantities of the reagents are properly adjusted: The addition of sodium chloride solution (an electrolyte) to a colloidal solution of gold chloride causes a precipitation of the gold chloride, with characteristic changes in the color of the solution. The colloids in normal cerebrospinal fluid exert a protective action on the colloidal gold chloride which prevents

this precipitation. In many pathological conditions the cerebrospinal fluid loses this protective action, and precipitation occurs.

Many technical difficulties are encountered in preparing a suitable colloidal gold solution. Patterson's method (1930) of preparing the solution as modified by W. C. Williams (1935) is now used by the U. S. Naval Medical School and has given great satisfaction.

#### Reagents

- 1. Distilled water used in the preparation of the stock solution and reagents should be triply distilled. A block-tin or resistant-glass distilling assembly must be used. To the first distillate is added sodium carbonate (1 Gm. per liter) to remove all traces of ammonia. Collect only the middle 80% of the distillate, discarding the first and last 10% of the second and third distillates.
  - 2. Gold chloride (Merck's Blue Label): 1% solution.
  - 3. Potassium oxalate (highest purity): 1% solution freshly prepared.
  - 4. Potassium hydroxide solution: 0.02 N (1.1222 Gm. per liter).
- 5. Hydrochloric acid solution: 0.02 N (0.7294 Gm. per liter; 1.7 cc. per liter, of 30% acid of sp. gr. 1.18).
  - 6. Edestin (Pfanstiehl Highest Purity): 1-2000 in 0.2% HCl.
  - 7. Sodium chloride solution: 0.4% solution.

## **Preliminary Titration**

- r. Set up 9 numbered test tubes.
- 2. In the bottom of these test tubes pipette increasing amounts of 0.02 N KOH as follows: No. 1, none; No. 2, 0.15 cc.; No. 3, 0.20 cc.; No. 4, 0.25 cc.; No. 5, 0.30 cc.; No. 6, 0.35 cc.; No. 7, 0.40 cc.; No. 8, 0.45 cc.; No. 9, 0.50 cc.
- 3. Place in a small flask 50 cc. of triply distilled water, 0.5 cc. of 1% potassium oxalate solution, and 0.5 cc. of 1% gold chloride solution.
- 4. Mix and add 5 cc. of this solution to each of the 9 test tubes. Immediately immerse in a beaker containing sufficient water at room temperature just to cover the level of the fluid in the tubes.
- 5. Place the beaker, protected by a plain wire gauze, over a bunsen burner, rapidly bring the water to the boiling point, and boil for 2 minutes.
- 6. Withdraw the tubes, place them in order in a rack, and allow to cool before reading.
- 7. Only one tube in the series represents the right amount of KOH to use in the preparation of the stock gold solution. It is the lowest tube in the series to give a bright red solution which, when viewed by reflected light, shows just the slightest sheen. The other tubes are eliminated by being too purple, too pale, or even colorless.
- 8. A simple multiplication by 200 gives the correct amount of 0.02~N~K()II to use in the preparation of 1000 cc. of the stock gold chloride solution.

#### Preparation of Stock Gold Solution

The stock solution is perfectly stable. The amount that may be made is limited only to the requirements of the laboratory and the quantity of reagents originally

prepared. When more than one lot of 1000 cc. has been prepared they should be pooled and stored in resistant-glass containers.

To prepare 1000 cc.:

- 1. Place in a 3 or 4 liter resistant-glass flask 1000 cc. of triply distilled water, add 10 cc. of 1% potassium oxalate solution, 10 cc. of 1% gold chloride solution and the amount of 0.02 N KOH determined by the titration (usually 40 to 50 cc.).
- 2. Bring to the boiling point rapidly without shaking. The solution will go through various color changes, colorless to pale blue, blue, purple, and finally a deep dark red.
- 3. Just before the boiling point is reached there is a sudden lightening of the solution which becomes a clear, bright red color. No further color change takes place, although the solution should be left over the flame until boiling starts. This lightening of the mixture is absolutely essential for a satisfactory solution. If this typical color change does not occur, there is something wrong with the reagents, their measurement, or the calculation of the amount of KOH used.
- 4. After the various lots are pooled and have stood over night, this stock solution is ready for the final titration.

#### Final Titration of Stock Solution

This stock solution is alkaline in reaction, and it is necessary to determine the amount of 0.02 N HCl to be added, as follows:

- 1. Set up 6 series of 10 test tubes each, as for the routine test on a spinal fluid.
- 2. To the first tube in each series add 0.9 cc. of 0.4% salt solution.
- 3. To each of the other tubes add 0.5 cc. of 0.4% salt solution.
- 4. Prepare a 1 to 2000 solution of edestin in 0.2% HCl.
- 5. Place o.1 cc. of this solution in the first tube of each series.
- 6. Make dilutions in the usual way. (Thoroughly mix the contents of the first tube, transfer 0.5 cc. to the second tube, mix, and so continue to the tenth tube, from which 0.5 cc. is discarded.)
- 7. Six 25 cc. portions of the stock gold solution are placed in 50 cc. flasks and numbered. These are acidified with increasing amounts of 0.02 N HCl as follows: Flask No. 1, 0.25 cc.; No. 2, 0.30 cc.; No. 3, 0.35 cc.; No. 4, 0.40 cc.; No. 5, 0.45 cc.; No. 6, 0.50 cc.
- 8. Each tube of series 1 then receives 2.5 cc. of acidified solution No. 1; each tube of Scries No. 2 receives 2.5 cc. of acidified solution No. 2; etc.
- The tubes are mixed by gentle shaking or rotating the individual tubes between the hands.
  - 10. The results are read after 18 to 24 hours.
- 11. Various degrees of reaction will be evident in the different series. That amount of 0.02 N HCl which just gives a moderately strong paretic type reaction (5555421000) is the correct amount to use in acidifying the stock solution just before use. Usually this is found in the third or fourth series, but this may vary with different preparations.

Since the 0.02 N KOH and HCl are not exact solutions that have been precisely standardized, it is possible that the desired paretic curve will not appear in the first six series. It is then necessary to set up additional series of tubes, increasing the acidity by 0.05 cc. of the 0.02 N HCl in each successive series over the sixth until the desired curve is obtained.

12 The stock container is labeled with the result of the titration.

13. The determined amount of acid should be added to the stock gold solution just prior to use, and only sufficient should be acidified for *immediate* use. The acidified gold solution is not stable in storage.

## Actual Test on Spinal Fluids

The following modification of Lange's Colloidal Gold Test has proved equal to the standard technique both in correctness and legibility, and is recommended in the interest of economy.

- 1. Place 11 chemically clean test tubes in a rack.
- 2. Into the first tube place 1.8 cc. of 0.4% sodium chloride solution and put 0.5 cc. into each of the remaining tubes.
- 3. Add 0.2 cc. of spinal fluid to the first tube and mix thoroughly. (We feel that the dilution will be more accurate if 0.2 cc. of fluid is diluted with 1.8 cc. of salt solution than if 0.1 cc. is diluted with 0.9 cc. of salt solution. If the latter is used, 1.0 cc. is not discarded from the first tube as directed in Step 4.)
- 4. Discard 1.0 cc. from the first tube, then transfer 0.5 cc. to the second tube and mix thoroughly. Transfer 0.5 cc. to the third tube, and continue until the tenth tube is reached, discarding 0.5 cc. from the tenth tube. The eleventh tube is used as a control.
  - 5. Add to each tube 2.5 cc. of colloidal gold solution.
- 6. Mix thoroughly and set aside for 24 hours. The readings are made and recorded as in the original test.

Note.—All glassware used in the preparation and titration of the solutions and in the spinal fluid test must be of resistant glass. It must be chemically cleaned with aqua regia, and thoroughly rinsed with tap, once-distilled, and finally, just before use, with triply distilled water.

The results are usually expressed numerically as follows:

Unchanged deep red	,
Bluish red	
Lilac or purplish	į
Deep blue	
Pale blue 4	
Colorless 5	

With a normal cerebrospinal fluid there is no change in color in any of the tubes. Three types of reaction occur:

- 1. Paretic type (first or left zone), in which the maximum change (complete precipitation) occurs in the first 4 or 5 tubes; e.g., 5555432100.
- 2. Luctic type (second or mid-zone), in which the maximum change (usually not complete precipitation) occurs in about the fourth and fifth tubes; e.g., 0124321000.
- 3. Meningitic type (third or right zone), in which the greatest change is in about the seventh and eighth tubes; e.g., 0001234421.

A minute trace of blood in a normal spinal fluid may give a meningitic curve. Any appreciable amount of blood renders the test entirely unreliable, as does even a slight degree of bacterial contamination.

Color change of slight degree occurring in the first 2 or 3 tubes alone is not significant. The tube used for collecting the fluid should be cleaned in the same way as those used in the colloidal gold test.

The chief practical value of the test is in the diagnosis of syphilis of the central nervous system, and particularly in differentiating general paresis from other types of syphilis. A diagnosis of paresis should not be based on a paretic curve alone, but the latter always indicates a grave type of infection which demands energetic treatment. However, a paretic curve (with a negative Wassermann reaction) occurs in some cases of multiple sclerosis.

Colloidal Mastic Test.—This test is useful to supplement the colloidal gold test, and it is a fairly satisfactory substitute for the gold test if circumstances make it impracticable to perform the latter. The mastic test has the following technical advantages: The mastic solution is easy to prepare (although not every lot is satisfactory). Ordinary distilled water suffices, and extraordinary precautions in cleaning apparatus are not necessary. A trace of blood or a slight degree of bacterial contamination does not interfere seriously, as it does with the colloidal gold test. The mastic test fails, however, to yield the different types of curves given by the colloidal gold test.

Mastic solution.—Dissolve 10 Gm. of gum mastic in 100 cc. of absolute alcohol and filter repeatedly until an opalescent solution free from turbidity is obtained. Just before use dilute 1.0 cc. to 10 cc. with absolute alcohol and pour this into 40 cc. of (ordinary) distilled water.

Diluting solution.—To 990 cc. of a 1.25% solution of sodium chloride add 10 cc. of a 0.5% solution of potassium carbonate.

Test.—(r) Set up a series of 10 small test tubes, adding to the first 1.5 cc., and to the others 1.0 cc. of diluting fluid. (2) To the first tube add 0.5 cc. of spinal fluid, mix, and transfer 1.0 cc. to the second tube. Mix, transfer 1.0 cc. to the third tube, and continue until the ninth tube is reached, discarding 1.0 cc. from this tube. The tenth tube serves as a control. (3) To each tube add 1.0 cc. of freshly diluted mastic solution. Shake and let stand 12 hours or more at room temperature. Then read.

The control tube is opalescent but remains clear. Five degrees of reaction are noted: milky turbidity, 1; cloudy with minute flocculi, 2; cloudy with coarser flocculi, 3; a coarse flocculent precipitate with a cloudy fluid, 4; and complete precipitation. 5.

A reaction of  $+^1$  or  $+^2$  in the first two or three tubes is of no significance. A  $+^3$  reaction is suggestive, and a  $+^4$  or  $+^5$  is definitely pathological. The maximum reaction practically always occurs in the first few tubes; the reaction is (qualitatively) "paretic" in type, regardless of the type of cerebrospinal syphilis present. Otherwise its significance is the same as that of the colloidal gold reaction. It is about equal to the latter in sensitiveness.

Methods for the determination of the non-protein nitrogen constituents, sugar and chlorides in spinal fluid are identical with those used for the Foiln-Wu tungstic acid blood filtrate, except that one fourth the quantities of sulphuric acid and sodium tungstate

## CEREBROSPINAL FLUID IN (After Fremont-Smith and Ayer in Jour

			Atter Premonesin		
Disease	Initial pres- sure: mm. of spinal fluid horizontal position	Rise on jugular com- pression	Appearance	Cells per emm.	Globulin, mgm.: 100 ee.
Lumbar Cisternal Ventricular	70-190* (Water manometer)	Prompt	Clear Colorless No clot	υ -5 ο 5 ο 5	
Blood plasma					
Brain abscess	+	N or de- layed in some cases.	Clear and colorless to turbid, clot ±.	ł	±
Acute purulent meningitis (Cocca intection).	+	±	Purulent; faint yellow; ± clot.	+ (Polys)	+
Acute anterior poliomyleitis	+	N	Slightly opalescent; rarely turbid; faint yellow ± delicate fibrin web.	Polys early shifting to lympho- cytes.	Slightly + ·
Bering 'ymm' squi'c chorio- (Aseptic lymphocytic men- ingitis).	+	N	Clear to hazy.	(Lymphs).	±
Tuberculous meningitis	+ '	N	Opalescent to turbid faint yellow, ± delicate fibrin web.	Lympho- cytes	+
Encephalitis lethargica A	N	N	N	N or slight	±
Encephalitis lethargica B Encephalitis lethargica St. Louis.				increase No polys.	
Acute syphilitic meningitis	+	N	Clear to turbid faint yellow, ± fibrin clot.	+	+
"Meningovascular syphilis"	±	N rarely delayed.	N rare fibrin clot with large fiber.	+	+
Progressive correlebringtons supported.	+	N	N rare fibrin clot.		+
Late inactive forms or insufficiently treated.	N	N	N	N	N
Alcoholism	+	N	Clear	(Lymphs).	N
Diabetes (coma)	Low	N	Clear.	Lymph.	N

N-Normal. +-Plus (positive).

## DIFFERENTIAL DIAGNOSIS nal of American Medical Association)

Protein, mg./100 cc.	Sugar, mg./ 100 cc.	Chlorides (as NaCl), mg./ 100 cc.	Non-pro- tein nitro- gen, mg./ 100 cc.	Gold sol.	Comment
15-40 10-25 5-15	50-75† 50-75† 50-75†	720-750	12-18	000000000	Sugar and chloride values apply to fasting nonfebrile individuals.
6,200-8,200	70-110	570-620	18-30		
Slight increase.	N or +	N	N	Variable	Polys nearly always present. If complicated by septicaemia or high fever chlorides may fall to low levels.
+	Low	土	N	Variable	Sugar may be but slightly decreased at outset. Often falls rapidly to under 10 mg. Chlorides rarely below 640 unless septicaemia is present. Meningococci found in smear and culture with difficulty; pneumococci, streptococci and staphylococci ensily.
Slight +	N	N or slight decrease.	N	Variable	In pre-paralytic stage polys may exceed 80 %—Rapid change to lymphocytes. With gradual increase in cells, protein increases for two to three weeks. Paralysis rarely present until marked lymphocyte increase (90 %).
±	N	N	N	Variable	Signs of manipulation with increase sterile, negative for tuberculosis. Disease benign in nature. Etiology: filtrable virus. Often mistaken for tuberculous meningitis.
+	N or low	Very low chlorides almost diag- nostic.	N	Variable	Excess fluid. Polys apt to be increased very early and in infants. Chlorides usually below 6.40 mg./100 cc. Tubercle bacilli found in clot or sediment. Guinea pig inoculation positive.
N or slight increase.	N	N	N	Variable	Sugar is normal unless blood sugar is elevated. Over 50 % of cases have normal cell count. Others rarely exceed 60 cells. Protein increase when present is slight—rarely reaching 100 mg./100 cc.
+	N	Slightly low.	N	Strong reaction Zone variable.	Wassermann reaction nearly always positive.
+	N	N	N	Variable	Wassermann reaction nearly always positive.
+	N	N	N	Paretic or luetic.	Wassermann reaction always strongly positive. (Includes tabes 80 %, paresis 100 %, and optic atrophy 80 %).
±	N	N	N	Weak luetic ±.	Wassermann reaction weakly positive or negative.
N	N	N	N	N	Alcohol present in the spinal fluid.
N	Marked	N	N	N	Increased sugar in sp. fl.

<sup>±—</sup>Plus-minus normal or increased.

\* Divide by 13.5 to convert to mm. of mercury.

† Depends upon blood sugar level.

suffice for precipitation of the protein. For the estimation of sugar only, dilute 1.0 cc. of spinal fluid with 4 cc. of water and proceed as with the blood filtrate.

#### Bacteriological Examination

If meningitis is suspected, or if the fluid is cloudy, a portion should be inoculated immediately on blood agar (or dextrose brain broth) and the tubes kept at body temperature until placed in the thermostat. If the fluid is cloudy films should be made and stained by Gram's method, and if indicated, for tubercle bacilli.

In addition to the meningococcus, many other species of bacteria may cause a meningitis, most frequently pneumococci, streptococci and staphylococci. The meningitis may be haematogenous in origin, one manifestation of a general septicaemia, or it may result from direct extension from some localized area of infection, most frequently in the middle ear, mastoid or paranasal sinuses.

If tuberculous meningitis is suspected and a clear or opalescent fluid is obtained, a tube of fluid may be put in the ice box (Levinson recommends room temperature), kept undisturbed, and examined after 24 hours. If a fine filmy clot has formed, this should be fished out carefully with a platinum needle, spread over a small area on a slide and stained for tubercle bacilli in the usual way. They can be demonstrated without great difficulty in a large majority of the cases.

The method of Sheo-Nan Cheer has proved satisfactory. To 5 cc. of spinal fluid in a centrifuge tube add 95% alcohol drop by drop, shaking the tube, until the fluid looks milky, or until one third to one half its volume of alcohol has been added. If necessary add a drop or two of diluted egg albumin. Centrifugalize until clear, decant the fluid, take up the sediment with a capillary pipette, make films and stain.

If no tubercle bacilli are found, a portion of the clot or sediment should be inoculated on suitable media, and a guinea pig inoculated.

If benign lymphocytic choriomeningitis is suspected, several mice should be inoculated intracerebrally, or guinea pigs subcutaneously.

#### Animal Parasites

Trypanosomes may be found in the spinal fluid in the advanced "sleeping-sickness" stage of trypanosomiasis. The lymphocytes are increased.

Trichinella embryos have been found in the spinal fluid.

## EXUDATES AND TRANSUDATES

The serous cavities normally contain only minute amounts of fluid. Large amounts of fluid may accumulate in them under pathological conditions. Such fluids are divided into two classes: (1) Transudates, which form as a result of circulatory stasis or obstruction. (2) Exudates, which result from inflammatory processes. It is practically important and often possible to differentiate between the two conditions by a study of the characteristics of the fluid.

If possible several ounces of fluid should be collected in sterile condition, and a portion should be mixed with a little potassium oxalate or 2% sodium citrate solution to prevent clotting. The following examinations should be carried out:

1. Gross Appearance.—Transudates are clear and transparent or opalescent, and pale yellowish in color. Exudates are usually more highly colored and more turbid. They may vary from serous (slightly cloudy) or serofibrinous to purulent; or they may be haemorrhagic or chyliform.

A trace of blood is common as a result of trauma of the puncture. A haemorrhagic fluid is frequently obtained in cases of malignant disease, but may occur also in acute tuberculous infections, and in a variety of other conditions (trauma, haemorrhagic diseases, aneurysms, etc.).

- A true *chylous fluid* (from which the fat can be extracted with ether) results from obstruction to large lymphatic channels (filariasis, occasionally cancer or tuberculosis of the lymph glands, etc.).
- 2. Specific Gravity.—This may be determined with an ordinary urinometer. Transudates are under 1.018 and usually under 1.015; exudates are usually over 1.018.
- 3. Protein Content.—The amount of protein in the fluid may be determined by means of the Esbach tube, as described for urine, first diluting the fluid I to 10 with water and acidifying; or the sulphosalicylic acid method may be used. Transudates contain less than 30 Gm. (and usually less than 25 Gm.) per liter; exudates, usually 30 Gm. or more. Transudates do not clot. Exudates contain fibrinogen and often clot on standing. Exudates differ also in containing "sero-mucin" ("nucleo-albumin").

Rivalta's test for sero-mucin.—To 100 cc. of water in a graduate add 2 drops of glacial acetic acid. Let a drop of the fluid to be tested fall into the dilute acid. A positive reaction is indicated by the appearance of a bluish-white cloud.

Moritz adds two drops of 5% acetic acid to the fluid. A heavy cloud-like precipitate indicates an exudate. A transudate may show a slight opalescence.

4. Cell Count.—The *total cell count* is made with a haemacytometer. In the case of serous fluids with relatively few cells, the fluid is used undiluted or mixed with a little staining solution, as described for spinal fluid. Cloudy or purulent exudates should be diluted in a leukocyte pipette as in making a blood count.

To make a differential count, centrifugalize a portion of the fluid as soon as possible after securing it, decant the fluid, resuspend the sediment in the drop remaining in the tube, and make thin films, using the same technique as in making blood films. Films may be stained by Giemsa's or Wright's stain, or they may be fixed by heat (flaming alcohol) and stained with haematoxylin and eosin. The cells degenerate rapidly if the fluid is allowed to stand and then stain poorly. In purulent exudates the leukocytes often show marked toxic-degenerative changes. If the films are thick and the cells rounded up and not well spread out, it is difficult or impossible to identify them. The interpretation of such films is often difficult and requires considerable experience. It is impossible to make satisfactory counts or films from clotted fluid.

Transudates and Exudates.—In transudates the cell count is low, usually under 100, and nearly all are large mononuclear mesothelial cells. These may be in plaques. A few lymphocytes may be present.

In tuberculous exudates the cell count is moderately high, usually from 100 to 1000 or more, and nearly all the cells are lymphocytes. A few large mononuclear cells may be present, and in acute cases some leukocytes.

In exudates due to other organisms the cell count is much higher, often many thousands, and nearly all are polymorphonuclear leukocytes.

A serous exudate may develop without actual infection of the cavity as a response to some localized infection in the immediate vicinity (e.g., a lobar pneumonia or a perirenal abscess). Such fluids show a more moderate increase in cells which are largely leukocytes, and yield sterile cultures.

Exudates due to malignant disease may contain tumor cells, but it is difficult to identify them with certainty. The most important points are the occurrence of clumps of cells, and the presence of mitotic figures. These are best demonstrated by fixing the moist films with some bichloride fixative and staining with haematoxylin and eosin. In some cases there are very large, vacuolated cells which have the appearance of a signet ring, but similar cells have been reported in chronic inflammatory processes.

More satisfactory results have been reported with Mandelbaum's method. The sediment from a considerable amount of fluid is collected by sedimentation and centrifugalization. To the sediment in the tip of the centrifuge tube add 10% formalin and fix for 24 hours. Then dehydrate, imbed in paraffin, section and stain with haematoxylin and eosin like a piece of tissue.

The differences between transudates and exudates are not always clear-cut. A transudate may acquire the characteristics of an exudate after it has become chronic, particularly after repeated tappings. It may become concentrated and show a high specific gravity and high protein content during the process of absorption. An exudate due to a mild chronic infection, particularly in the peritoneal cavity, may show a relatively low gravity and protein content, like a transudate. Stasis may play a part in the accumulation of such fluids.

5. Bacteriological Examination.—Cultures should be made on blood agar. If the fluid is cloudy or purulent, films should be stained by Gram's method, and with methylene blue.

In cases of acute peritonitis Wilkie pointed out that the examination of stained films of the exudate gives information of prognostic value. If bacteria are sparse and largely intracellular, the outlook is good. If they are numerous and largely extracellular, and if the leukocytes show degenerative changes, the prognosis is unfavorable.

If tuberculosis is suspected, cultures of the centrifuged sediment should be made and guinea pigs ino culated. Positive results may be expected in at least 50% of the cases from pleural fluids, less frequently from ascitic fluids. A portion of the sediment may be stained for acid-fast bacilli, but they are found only occasionally.

Synovial fluid resembles that from the serous cavities. The normal cell count is about 50, and 95% of the cells are mononuclears. The sugar and pH are practically the same as in the blood.

In chronic infectious (rheumatoid) arthritis cultures of the fluid are sterile. The cell count is increased, but usually under 5000, and less than 50% of the cells are polymorphonuclear leukocytes. The sugar and pH are slightly reduced (60 or less, about 7.0).

In acute infectious arthritis from which positive cultures are obtained the cell count is usually over 10,000, and over 50% are polymorphonuclear leukocytes. The sugar is under 45 mg. and the pH under 7.0.

Traumatic arthritis is suggested by the presence of red cells in the fluid and by an icterus index over 6.

In tuberculous arthritis with effusion organisms can practically always be demonstrated by culture or guinea pig inoculation.

## CHAPTER XXXIII

## CHEMICAL EXAMINATIONS OF THE BLOOD

SIMPLE accurate methods are now available for the quantitative estimation in small amounts of blood of most of its known important constituents. Alterations from the normal in the quantity of some of these substances occur in a wide variety of conditions, and their determination yields information of great practical value in diagnosis, prognosis and control of treatment. They are especially useful in diabetes, renal disease, coma of unknown origin, conditions associated with oedema or dehydration, and disturbances of acid-base equilibrium. However, an accurate interpretation of the results can be made only in conjunction with all the other available information concerning the patient. In general these analyses are of less value as direct diagnostic procedures than as a means of recognizing disorders of metabolism and of guiding therapeutic measures necessary to relieve them.

For practical clinical purposes, to avoid wasting time and effort, it is important to select those procedures which are likely to yield useful information in the disorder which is present or suspected, and not to follow blindly some fixed routine analytical procedure. Thus it is useless as a rule to determine the urea nitrogen or non-protein nitrogen in a patient with nephritis, whose renal function is known (by other tests) to be fairly good. It is rarely worth while to determine both urea nitrogen and non-protein nitrogen, since their figures usually parallel one another, and a rise in one has the same significance as a rise in the other. It is useless to determine creatinine if the urea nitrogen is normal. Determinations of the chlorides, plasma proteins or the CO<sub>2</sub> combining power are much more often helpful than those of uric acid. Tests which are indicated should be done accurately and preferably in duplicate.

The following table gives a summary of the normal figures and alterations which may be expected in various clinical conditions. The figures for average normal findings are taken from Peter's table (1934) with a few unimportant exceptions. The others have been compiled from various sources. The values are given in mg. per 100 cc. of whole blood (the usual system) or as millimoles (mM) or milliequivalents (mE) per liter, unless otherwise indicated. "Inc." and "Dec." signify increased and decreased, respectively; "N" signifies normal; "Var," variable. "To" qualifying a figure indicates that findings range from normal to that figure. Workers should notice the marked differences between the concentration of many of these substances in cells and in plasma and the effect that anaemia alone may have on the results of analyses if they are made with whole blood.

Collection of Blood.—To secure constant conditions the blood should be obtained, preferably by venepuncture, before breakfast in the morning. This is essential for sugar determinations. For most other substances roughly comparable results may be obtained if blood is taken four hours after a meal. Most determinations are made on whole blood, and coagulation is prevented by adding potassium oxalate, using not more than 3 mg. per 1 cc. of blood. For determination of calcium (and preferably of inorganic phosphorus) serum is used, and anticoagulants (particularly oxalate) must be avoided. Plasma is necessary for the determination of plasma proteins, and plasma or serum are much preferable for chlorides, carbon dioxide combining power, bases and cholesterol. They are equally satisfactory for all the other usual analyses (aside from haemoglobin and O) except that a larger volume of blood is required.

For determination of sugar and nitrogenous substances, blood may be taken in any convenient way and mixed with a little potassium oxalate in open bottles. For determination of blood gases, disturbances of acid-base equilibrium, and for accurate studies of chlorides and other electrolytes the blood must be protected from exposure to air. We recommend the apparatus shown in Fig. 208. Stasis must be avoided, and if a tourniquet is required, one should loosen it after the needle has entered the vein and wait until normal conditions are restored before gently aspirating the blood.

The following procedure (Peters and Van Slyke) is recommended for the preparation of the oxalate tubes. Make a 30% solution of potassium oxalate. If alkaline, recrystallize, and if necessary add enough oxalic acid to bring the reaction to a pH of 7.4 (to phenol red, see p. 877). To a series of tubes add with a graduated pipette 0.05 cc. of the solution (for 5 cc. samples) and 0.1 cc. (for 10 cc. samples). Hold tube horizontally, rotate until a thin film of oxalate solution is distributed over the walls, and dry without heating in a current of air.

After blood has been obtained the protein precipitation or other analytical procedures should be carried out without delay. If this is not possible, the blood should at once be chilled in ice water, and kept cold till used. In a hot laboratory the glucose content falls quite rapidly (after 1 to 2 hours), with simultaneous formation of lactic acid, due to the action of a glycolytic ferment which is eliminated with the proteins. Most other determinations (except acid, pH, and CO<sub>2</sub>) show less change during the first six or eight hours, but the longer the analysis is delayed, the greater are the errors encountered. Blood can be preserved 2 or 3 days at ordinary temperatures for glucose and non-protein nitrogen determinations, if it is sterile, and if 10 mg. sodium fluoride and 1 mg. thymol per cc. are added to prevent coagulation and inhibit ferment activity.

For all the determinations that follow, several different procedures are available which are accurate and satisfactory, and selection of a single method has been difficult. Those recommended are not necessarily the newest, or those capable of the greatest precision. We have tried to select methods of established reliability, which give results sufficiently accurate for all clinical purposes, which are simple to carry out, and which can be performed with solutions which are simple to prepare and are relatively stable.

For the determination of glucose and the non-protein nitrogenous constituents of the blood the *Folin-Wu system* of analysis is satisfactory, and the following procedures utilize their tungstic acid filtrate and largely follow their technique.

Precipitation of Protein. (Folin-Wu, Haden).—(1) In a clean dry flask pipette (preferably with a Folin pipette) one volume of well mixed blood (10 cc. for a complete analysis). (2) From a graduate or burette add slowly with constant stirring 8 volumes of N/12 sulphuric acid. (3) Add 1 volume of 10% sodium tungstate solution; shake

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	Non- protein nitrogen	Urea nitrogen	Uric acid	"Creati- nine"	Glucose (true)	Sodium (plasma)	Potassium (plasma)	Calcium (plasma)	Inorganic phos- phorus (plasma)	Chloride as Cl (plasma)
Normal: Whole blood Range. Plasma or serum.	39 25-39 29	18 12-18 19	2-4 3	1.2 1.2 1.2	96 001–09 *98	172 310 140-145	197 18 5 mM	5.6 10 9-11.5	3.4°5 3.4°5	285 362 352-380
Cells. Anaemia (50°6). Diabetes moderate. Diabetic coma	94 6	17 18 15–30	~ ~ · · · · · · · · · · · · · · · · · ·	2 : 1 : 2 2 : 1 : 4 : 1	75 91 150-300 300-1000	mM Trace 241	420 IO7	5 mE 0 7.8		99-108 mM 192 323 Dec.
Fancteduc disease Arteriosclerosis, Acute nephritis. "Nephrosis"	40-250 N-Inc.	20-200 N-Inc.	3-5	2-10 1-4	Inc. N-Inc. Inc. N-Inc.	Var.	: : : : : : : :	N Dec.	N N-Inc.	N . N-Inc. N-Inc.
Contracted kidney. Uraemia	90-350 90-350	To 250 To 300	To 10 Inc.	To 16 Inc.	Inc. Inc.	To 128	×	N-Dec. Dec.	To 20 To 20	To 120 mM Var Var
Bichloride poisoning.  Gout.  Hyperthyroidism.  Myroeparathyroidism.  Hyperparathyroidism.	To 350 N-Inc. N-Inc.	To 300 N-Inc. N-Inc.	To 16 4-10	To 3.5	Inc. Inc. Dec.			N-Dec. N-Inc. To 18 To 7	Dec. Inc.	Dec. N-Dec.
Addrawn s  Addrawn Addrawn Belampsia Necross of Ilver. Infestinal obstruction Permicros anaema Obstructive jaundice.	Inc. To 70 To 45 Inc. To 300	Inc. N-Inc. To 25 Dec. Inc.	N-Inc. Inc. N	To 2.5	Dec. N-Inc. Dec.	D D C C C C C C C C C C C C C C C C C C	Inc.	Dec. N. N. Dec.	Inc.	Dec. (To8o mM) N Dec.
		~				-	_			

\*70 to 120 by the usual methods.

Globulin, Haemo- " Haemo- " Globin, Glasma)	15.6 2.0 2.0-3.5	35	Var. Dec. Dec. Dec. Dec.	Dec.	Ļ
Albumin, % % % % % % % % % % % % % % % % % % %	3.8-5.2		To 1.0	:	
Total protein, % (plasma)	19.5 7.0 6.3-8.0	35 IS.2	To 3.5	:	
CO:— Vol. % (bicarbonate) ate) (plasma)	58 68 50-75	727-33 mm 45 63 To 10	N-Dec. N-Dec. To 12 To 25	:	
Diastase (units)	8-64	Inc.	Linc:	Dec.	:
Bilirubin (plasma)	0.I-0.4			:	:
Fatty acids	290-410 355 190-420	Inc.	Inc. Inc. Inc.	Dec. Inc.	:
Choles- terol	140-210 207 140-210	Inc. To 800	N Inc. To 900 Var. N-Dec. N-Inc.	To 80 To 1350	Dec.
	Normal: Whole blood Plasma or serum Range.	Cells. Anaemia (50 %). Diabetes moderate. Diabete coma. Pancrate disease.	Artenisclerosis. Acute nephritis. "Nephrosis. "Nephrosis. "Ordiracted kidney. Uraemia. Bichloride poisoning.	Gout Hyperthyroidism Myxoedema Hyperparathyroidism Hypoparathyroidism	Addison's disease. Pneumonia

thoroughly. If the precipitation is complete the color becomes chocolate-brown; on shaking, it gives a ringing metallic sound; and it shows practically no foam. (4) After 10 to 20 minutes filter through a dry ammonia-free filter large enough to hold the entire quantity. Refilter the first few drops if necessary. The filtrate must be perfectly clear and colorless. It should be acid to litmus but not to congo red. If brownish, add one drop of 10% H<sub>2</sub>SO<sub>4</sub>, shake for a few minutes, and continue until a clear, faintly acid filtrate is obtained. The filtrate may be kept for 24 hours in the ice box, with a drop of xylol as preservative.

For plasma or serum, dilute both the  $N/12~H_2SO_4$  and sodium tungstate solution with an equal volume of water, and proceed as above, using the diluted reagents.

 $N/_{12}$  sulphuric acid.—Add 2.5 cc. of concentrated sulphuric acid to 1 liter of water. Check the strength of the solution with an accurately standardized alkali solution (see p. 874).

Sodium tungstate reagent.—Dissolve 100 grams of pure sodium tungstate in 1 liter of ammonia-free water. Filter if necessary. The reaction should be faintly alkaline, but such that less than 0.4 cc. N/10 HCl will render 10 cc. neutral to phenolphthalein. If necessary add acid or alkali sufficient to bring it to this point.

Check both solutions by showing that they yield a suitable filtrate with normal blood. If tightly stoppered they keep indefinitely.

## Non-protein Nitrogen and Urea Nitrogen

Non-protein Nitrogen (Folin-Wu).—All reagents must be of the highest purity, and free from N. Ammonia-free water must be used for all these determinations. Other tests requiring the use of ammoniacal solutions should not be carried out in the same room in which nesslerization procedures are performed.

(1) With a volumetric pipette put 5 cc. of blood filtrate in a dry Pyrex ignition test tube (25 × 200 or 250 mm.) graduated at 35 and 50 cc. (The use of a wet tube causes troublesome bumping.) (2) Add I cc. of diluted acid digestion mixture and a dry quartz pebble. (3) Boil vigorously over a microburner, shaking constantly, until dense white fumes fill the tube. (4) Quickly transfer the tube to a stand, cover mouth of tube with a watch glass, and continue heating just enough to maintain visible boiling for two minutes, or until solution is clear. (5) Let cool in place for 90 seconds (not longer). (6) Add cautiously about 25 cc. of water, at first a few drops at a time. (7) Cool to room temperature, and fill to the 35 cc. mark with water. (8) In a 50 cc. volumetric flask put 3 cc. of the working nitrogen standard solution, containing 0.15 mg. of nitrogen, I cc. of diluted acid digestion mixture, and about 25 cc. of water. (9) To each, I or 2 drops of gum ghatti solution may be added. (10) Nesslerize both solutions at the same time, adding to each 15 cc. of Nessler's solution, shaking constantly while adding the solution; dilute the standard to the 50 cc. mark, and mix. (11) Stopper the tube containing the unknown with a clean rubber stopper, mix, and if it shows a whitish turbidity, due to etching of the glass, centrifugalize until clear. (12) Compare in the colorimeter, reading the standard against itself.

Calculation:

 $\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 30 = \text{mg. NPN per 100 cc. of blood}$ 

If the sediment after centrifugalization is reddish, if the solution is not perfectly clear, or if either solution clouds before the reading is completed, the nesslerization is a failure and the test must be discarded. Clouding can often be prevented by adding 1 or 2 drops of gum ghatti solution to each tube before nesslerization, but in our experience this is rarely necessary. If the color of the unknown is much darker than that of the standard, the test must be repeated, using less of the filtrate.

Clouding of the solution after nesslerization may be due to improperly prepared solutions, especially an improper amount of alkali in the Nessler's solution. Check: 20 cc. of N/1 HCl should be neutralized (to phenolphthalein) by from 11 to 11.5 cc. of Nessler's solution. It may also be due to the following technical errors: (1) Inadequate heating, covering the mouth of the tube too soon, or stopping before the solution is entirely clear. (2) Overheating, by boiling too long or too vigorously after covering the tube. (3) By diluting with water too soon, or more often, too long after heating is completed. (Considerable sputtering is to be expected when the first drops of water are added). (4) By not keeping the solutions well mixed while the Nessler's is being added. (5) By waiting too long before making the readings.

Solutions. Acid digestion mixture.—Mix 300 cc. of 85% phosphoric acid with 100 cc. of concentrated nitrogen-free sulphuric acid, and 50 cc. of 5% copper sulphate solution, put in a tall cylinder, seal tightly to exclude ammonia, and let stand several weeks till the precipitate of CaSO<sub>4</sub> has settled out. Decant or siphon off the clear fluid.

Diluted acid digestion mixture (working solution).—To one volume of the clear solution above, add an equal volume of water.

Nessler's solution. (A) Iodide solution.—In a 500 cc. Florence flask put 150 Gm. of potassium iodide, 110 Gm. of iodine, and 100 cc. of water, and 140 to 150 Gm. of metallic mercury. Shake vigorously and continuously (for 7 to 15 minutes) until the red color of the solution begins to get paler. Then cool under running water, still shaking, until the color turns greenish. Decant the fluid from the mercury, wash liberally with water, add the washings to the original fluid, and dilute the mixture to 2 liters. It should be clear. If not, clear by sedimentation and decantation.

(B) 10% sodium hydroxide solution.—This should be carbonate free, prepared as described on p. 874. The strength must be checked by titration (1 cc. of 10% NaOH is neutralized by 25 cc. N/10 HCl), and il necessary adjusted so that the error is less than 5%.

The Nessler's solution (working solution) is made by adding to 700 cc. of the 10% NaOH, 150 cc. of the double iodide solution, and diluting to 1 liter with distilled water. Mix, and clear by prolonged sedimentation and decantation (or if needed at once by centrifugalization). This solution must be kept tightly sealed to exclude ammonia, and pipettes used to withdraw solution should be labeled and used for no other purpose. If properly stored it keeps indefinitely.

Standard nitrogen solution (stock).—Dissolve exactly 4.716 Gm. of dry ammonium sulphate, of the highest purity and pyridine-free, in 1 liter of water. This contains one gram of nitrogen, hence 1 mg. per 1 cc. (For the average laboratory we advise the purchase of the specially purified pyridine-free preparation.)

Standard nitrogen solution (working).—Dilute exactly 5 cc. of this solution to 100 cc. with water. 1 cc. contains 0.05 mg. If tightly stoppered these solutions keep indefinitely.

Ammonia-free water.—The water used should give no appreciable color with Nessler's reagent. If it does, it must be freed from ammonia by adding a little sulphuric acid, and

redistilling from a glass flask. The water must be protected from exposure to ammoniacontaining air during the collection, by receiving it in a flask the vent tube of which is protected by a guard tube containing a little dilute sulphuric acid.

Urea Nitrogen.—(1) In a large Pyrex ignition tube put 5 cc. of blood filtrate. (If the tube has contained Nessler's solution, it must be washed out with nitric acid and water first). (2) Add 3 drops of the buffer phosphate mixture. (3) Add 1 cc. of urease solution (or a urease tablet, or a piece of urease paper, of known activity). (4) Put in a water bath at 50°C. for 5 minutes, or at 37° for 15 minutes (or longer). From this point several alternative procedures are available.

(A) Looney's modification of Karr's method by direct nesslerization.—(5) Add about 15 cc. of water, and 4 drops of gum ghatti solution. (6) In a 50 cc. volumetric flask put 3 cc. of working standard nitrogen solution (containing 0.15 mg. of N), 2 cc. of urease solution, 8 drops of gum ghatti solution, 6 drops of buffer solution, and about 30 cc. of water. (7) At the same time nesslerize both solutions by adding 2.5 cc. of the working solution to the blood filtrate, and 5 cc. to the standard. (8) Dilute each to the mark (the standard to 50 cc., the unknown to 25 cc.). Mix and read in the colorimeter without delay.

Calculation:

Reading of standard X 15 = mg. urea N per 100 cc. blood.

To express as urea, multiply this figure by 2.14.

The gum ghatti is necessary (and usually effective) to prevent clouding of the solution after nesslerization. If clouding does occur, the test must be repeated using one of the following alternative procedures.

(B) Wakeman and Morrell.—(5) Add about 1 gram of dust-free permutit and let stand about 5 minutes, shaking gently several times. (6) Clear the fluid by sedimentation (or centrifugalization if necessary), and decant or pipette off the fluid and discard. (7) Wash once with water, and similarly remove the fluid. Great care must be taken in these two steps not to lose any of the powder, or some of the nitrogen will be lost. (8) Add a few cc. of water to the powder, and 1 cc. of 10% sodium hydroxide and let stand a few minutes. (9) Add to a 50 cc. volumetric flask 3 cc. of working nitrogen standard solution, 2 cc. of 10% sodium hydroxide solution, and about 30 cc. of water. (10) Add to the blood filtrate water to about 20 cc. (11) Nesslerize both solutions by adding 2.5 cc. to the blood filtrate and 5 cc. to the standard. (12) Fill to the mark, mix, and read in the colorimeter. The calculation is the same as in (A).

(C) Folin-Wu.—The ammonia is liberated from the filtrate by alkalinization, and transferred to a tube containing dilute acid by aeration, or (as we prefer) by distillation. (For accuracy this is preferable to the preceding methods.) (5) In a similar large tube graduated at 25 cc., put 2 cc. of N/20 HCl. (6) Connect the two large ignition tubes by means of a glass tube 16 to 18 inches long, bent at a point 4 to 6 inches from one end so that the segments are nearly parallel to each other. The shorter end is connected with the tube containing the filtrate by means of a rubber stopper with a single perforation. The long segment of the glass tube is similarly connected with the receiving tube, and it must reach well below the surface of the acid in this tube. Λ bulb blown in this segment above the stopper helps to prevent back suction. (Λ long 5 cc. volumetric pipet may be used.) A groove should be cut in the stopper of the receiv-

ing tube to allow escape of air. (7) Into the filtrate tube place a long coil of heavy iron wire, to lessen bumping and frothing. It should extend to the bottom of the tube, and to a point an inch or two below the stopper. An antibumping tube may be used if necessary. Attach the tubes to a stand by means of clamps. It is convenient but not essential to cool the receiving tube in a breaker of cold water. (8) With the apparatus in position, loosen the stopper in the filtrate tube, and add 2 cc. of saturated borax solution. Do not add oil or other foreign substance to prevent frothing. Immediately replace the stopper tightly. (9) Heat gently with a microburner, by hand, so that a uniform moderately rapid stream of air bubbles and later of steam is driven into the receiving tube. It should be so adjusted that steam begins to emerge after 3 minutes. Continue for one minute longer, then loosen the stopper of the receiving tube, and lower the tube so that the opening of the glass tube is above the level of the fluid; and heat for one minute longer. (10) Rinse the end of the tube with water, cool, and add water to about 20 cc. in all. (11) In a 50 cc. flask put 3 cc. of working nitrogen standard solution, (containing 0.15 mg. N), and about 35 cc. of water. (12) To each, 1 or 2 drops of gum ghatti solution may be added. (13) Nesslerize at the same time, adding 2.5 cc. to the distillate, and 5 cc. to the standard solution. (14) Dilute both to the mark, mix, and read in the colorimeter. The calculation is the same as in (A).

Both of these procedures eliminate disturbing substances so that clouding after nesslerization practically never occurs. Some dexterity is required in the heating, to avoid overheating (which will cause some of the filtrate to froth over into the receiving tube) or too abruptly lessening the heat, which will cause acid in the receiving tube to be sucked back into the filtrate. If the latter happens, more acid may be added to the receiving tube, and the distillation repeated.

Aeration usually gives equally satisfactory results, but some substance like caprylic alcohol is usually necessary to prevent frothing; traces of this are carried over, and occasionally cause clouding after nesslerization.

Solutions. Buffer phosphate mixture.—To a 100 cc. volumetric flask add 14 Gm. of sodium pyrophosphate (Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>: 10H<sub>2</sub>O) and 2 cc. of 85% phosphoric acid, dissolve in water, dilute to the mark, and mix. It is essential for complete conversion of the urea.

Urease solution.—In a 250 cc. flask put 15 Gm. of Jack bean meal, 5 Gm. of permutit, and 100 cc. of 16% alcohol. Shake for 15 to 30 minutes. Filter through ammonia-free paper. This solution keeps active for two to four weeks on ice. Where only occasional tests are made, it is advisable to cut heavy porous filter paper into strips one inch wide, saturate them in the solution, dry at room temperature, and cut into 1.5 inch lengths. These keep active for many months. Reliable urease tablets can be purchased. The activity of such preparations should be checked occasionally.

Gum ghatti solution.—This is used to stabilize colloidal suspensions. Fill a liter cylinder with distilled water. Put 20 Gm. of gum ghatti in tea ball, and suspend it just below the surface of the water, letting it extract over night. Add 1.0 Gm. benzoic acid dissolved in 10 cc. of alcohol and shake. Clear by sedimentation if necessary. The solution keeps for months on ice.

Saturated borax solution.—Shake up an excess of pure sodium borate in distilled water, and keep in a tightly stoppered bottle.

Permutit.—This is a finely granular insoluble sodium aluminium silicate, which absorbs ammonia (with liberation of sodium) in neutral or acid solutions, and liberates the ammonia when sodium hydroxide is added. Absorption is complete only in dilute

solutions, and with an excess of permutit. To free the permutit from fine dust-like particles which sediment very slowly, repeatedly shake up the permutit in a flask with distilled water, wait a moment till the coarse particles have settled, and pour off the cloudy supernatant fluid. Permutit which has been used can be reclaimed (if it has not been in contact with Nessler's solution) in the following way. Wash several times with water. Add 10% sodium hydroxide, shaking occasionally for an hour or so. Wash with water several times, then with 2% acetic acid, and again several times with water. Dry in air without heating. Keep in bottle scaled with wax or paraftin.

The salivary index of renal function (Hench and Aldrich, 1923) serves as a rough gauge of the degree of nitrogen retention and is useful if for any reason accurate blood analyses can not be carried out. It is not an adequate substitute for them.

(1) Rinse the mouth thoroughly with water. (2) Collect 5 to 10 cc. of saliva and discard. (3) Collect another specimen and transfer just 5 cc. to a small flask. (4) From a small burette add, drop by drop, a 5% solution of HgCl<sub>2</sub> (chemically pure and accurately prepared), stirring vigorously, until a drop of the mixture, when added to a drop of saturated sodium carbonate solution in a white porcelain dish, turns reddish-brown within three seconds. (4) The salivary index ("mercury-combining index") is 20 times the number of cc. of HgCl<sub>2</sub> added.

The normal salivary index is from 30 to 50.

The probable blood  $urea = (1.45 \times salivary index) - 34$ .

Blood Urea Retention Test.—Hench and Aldrich (1926) have adapted the same procedure to blood. (1) To 7 cc. or more of oxalated blood add an equal volume of 10% trichloracetic acid, shake, and after 5 minutes or more centrifugalize. Titrate 5 cc. of supernatant fluid with 5% HgCl<sub>2</sub> as in the preceding test. The mercury-combining power per 100 cc. of blood is 40 times the number of cc. of HgCl<sub>2</sub> solution added. For normal blood this is 70 to 100. To obtain the probable blood urea, from this figure subtract 60, which represents the mercury-combining power of substances other than urea in the filtrate. Although figures obtained by this method can not be regarded as more than approximations, Nicholson found that the difference between them and the results of the usual urease methods was not of practical clinical significance.

Significance.—Normal individuals on an average diet show a fasting non-protein nitrogen of from 20 to 35 mg. per 100 cc. of blood, and a urea nitrogen from 10 to 20 mg. Variations from these figures may be brought about by the interaction of four major factors. (1) The amount of protein in the diet. (2) The occurrence and rate of destruction of body protein. (3) The volume of urine excreted. (4) The integrity or impairment of renal function, particularly as shown by the concentrating power, and capacity to excrete urea. The relative importance of the first three of these factors is not generally appreciated.

In normal individuals a very low protein diet which is adequate in caloric value may lower the N.P.N. to 13 mg. %. A reduction of the N.P.N. to normal figures may often be brought about in a similar manner in patients with marked (but not extreme) impairment of renal function. (The undesirability of this procedure is discussed later.) An abundant fluid intake with free diuresis may lower figures somewhat in a normal individual, and has a potent effect in preventing accumulation of N.P.N. in conditions

which tend to produce it. Slightly low figures often occur in the later months of pregnancy. On the other hand a diet abnormally high in protein, if maintained, may cause a moderate increase above these figures, if the fluid intake is not abundant.

Destruction of body protein occurs in a great variety of pathological conditions, and if marked in degree, may cause a substantial rise in N.P.N. if the urine volume is small. When associated with marked oliguria, the N.P.N. may reach levels as high as those usually seen in extreme renal insufficiency. Among the more important conditions in which destruction of body protein may lead to an increase in N.P.N. without significant renal injury may be mentioned: starvation, especially with fluid restriction; poisoning with various drugs; sometimes after the administration of potassium iodide; surgical shock; acidosis; peritonitis; severe infections of all types; intestinal or pyloric obstruction; dehydration from any cause; intractable vomiting or diarrhoea, as in cholera or dysentery; acute yellow atrophy of the liver; and the terminal stage of Addison's disease. In acute yellow atrophy (and in some cases of intestinal obstruction) the urea nitrogen has been reported to be relatively less clevated than the N.P.N., or in the terminal stage greatly reduced, the amino acids being greatly increased. In practically all other conditions the increase is mainly in the urea fraction.

In such infections as pneumonia a N.P.N. as high as 70 mgs. % has been reported, without evidence of renal injury and with an essentially normal, concentrated urine. By increasing the fluid intake and securing divresis, the figure may be brought to normal in two or three days, without any other notable change in the patient's condition. It is especially in dehydration associated with obstruction to the gastro-intestinal tract and with vomiting of HCl that excessively high figures have been observed. Peters and Van Slyke mention a case of pyloric obstruction with a N.P.N. of 202 mgs. % and Volhard reported almost as high figures in severe diarrhoea. If diuresis can be established by parenteral administration of salt solution, such figures can be speedily lowered. The persistence of a high N.P.N. after an operation for relief of peritonitis or intestinal obstruction (if renal function as determined by phenolsulphonephthalein excretion is good) is regarded as an unfavorable sign. The height of the N.P.N. is one indication of the devastating effect of dehydration in such conditions, and repeated observations are a helpful guide in treatment, although less significant than the fall in chlorides (and rise in CO<sub>2</sub>) which accompanies the rise in N.P.N. and which is probably its direct cause. High N.P.N. figures are rarely long maintained in such conditions, since the latter usually terminate quickly, either with death or recovery. (See section on blood chlorides and acid-base equilibrium.)

Chronic passive congestion, if uncomplicated, causes little or no rise in N.P.N., unless it is extreme. If it develops in the course of an infection, or in an individual with some degree of renal injury, it may precipitate a marked and rapid rise in N.P.N. There may be a sharp transient rise in N.P.N. during or immediately after a diuresis which has eliminated an oedema (from any cause), probably because the water of the oedema fluid can be excreted faster than the N.P.N. substances it contains, and the latter temporarily accumulate in the blood.

The fact that the N.P.N. may be elevated in *nephritis* with renal insufficiency is now known by every one. It is not so generally realized that this is a late symptom, and of no value in the early diagnosis of nephritis. In an otherwise normal individual the removal of one kidney

causes little or no rise in N.P.N. A marked and sustained rise occurs only after about 3/4 of the kidney tissue has been removed or destroyed. It is obvious that focal lesions must be very extensive before they will cause a rise in N.P.N., unless associated with infection or some other process that causes a toxic destruction of protein. About the same ratio applies in the case of diffuse glomerular nephritis; about 34 of the renal function is destroyed before a marked rise in N.P.N. occurs. The rise in N.P.N. is one measure of the degree of renal insufficiency present, but it is not a direct indication of the type of lesion in the kidneys. However, the N.P.N. is not often elevated in cases of nephritis of the "nephrotic" type, and high figures most often occur at the height an acute attack of glomerular nephritis, and in the advanced stages of chronic glomerular nephritis, and contracted kidney due to arterial disease. In the terminal stages very high figures are usually found, 100 to 300 mgs. % or even higher. If one can exclude some transient remediable complication which may raise the N.P.N., a figure over 100 mgs. 70 makes the immediate outlook grave. However, it is not the high N.P.N. in itself which immediately causes uraemia. Occasionally uraemia occurs in cases with a N.P.N. only slightly elevated, and it may be delayed for weeks or months in cases with very high N.P.N. figures. It is important that such patients receive a diet adequate in caloric value, and containing sufficient protein to prevent wastage of body protein, regardless of what the N.P.N. may be. Excessive protein restriction is harmful, even though it is more effective in lowering the N.P.N. The nature of the supposed toxic substance that causes uraemia is still a complete mystery. It is quite possible that some of the symptoms may be due to leakage of essential substances through the damaged kidneys.

It is evident from these facts that valuable information can be obtained from a determination of either the N.P.N. or urea nitrogen in the blood, but that no accurate conclusions as to the significance of a high figure can be drawn without considering all the other available information about the patient, including the nature of his diet, the volume of urine, and the presence of infection, circulatory failure, or other significant complications. To determine both is superfluous. Urea N must be determined if a urea clearance test is to be done, and the N.P.N., if plasma proteins are to be estimated or if the liver is damaged. Otherwise it is immaterial which procedure is chosen. The significance of the N.P.N. in nephritis is discussed further in connection with tests of renal function (p. 737).

#### Creatinine

Procedure (Folin).—(1) Transfer to 2 large test tubes 2 cc. and 4 cc. of working standard solution and dilute to 20 cc. (2) To a third tube transfer 10 cc. of blood

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filtrate. (3) To each standard tube add 2 cc. of 1% sodium picrate solution, and to the filtrate, 1 cc. (4) To each standard tube add 2 cc. of 2% sodium hydroxide, and 1 cc. to the filtrate. (5) Mix and let stand 30 minutes. (6) Compare in colorimeter. Calculation:

$$\frac{20}{\text{Reading}} \times \text{r}$$
 (or two if stronger standard is used) = mg. creatinine per 100 cc.

If the color is much darker than the stronger standard, dilute the unknown with an equal part of: water, 10 cc.; sodium picrate and alkali solution, each 1 cc.; and multiply by 4 in the calculation.

Standard creatinine solution (stock).—Dissolve 1 Gm. creatinine, or 1.61 Gm. creatinine zinc chloride in 1 liter of N/10 HCl. Creatinine working standard solution: Dilute 1 cc. of stock solution to 100 cc. with water. Best renewed frequently.

Sodium picrate 1% solution.

Sodium hydroxide 2% solution.

Significance.—It has been shown that the substance in blood which gives this color reaction is largely not creatinine, but this does not affect the practical significance of the reaction. The normal figures are from 1 to 2 mg. %. It is relatively little influenced by diet, or by extra-renal conditions which may increase the N.P.N. It is increased in cases of nephritis with marked impairment of renal function, rising according to Peters and Van Slyke, about in parallel with the urea nitrogen. Figures over 5 mg. % in advanced chronic nephritis usually indicate impending uraemia (see p. 737).

#### Uric Acid

Procedure (Benedict's method).—(This method is somewhat less accurate than the new Folin procedure, particularly when applied to filtrates from whole blood, but the solutions are simpler to prepare. It is preferable to use filtrates from plasma or serum.)

(1) In a large test tube put 5 cc. of blood filtrate and add 5 cc. of water. (2) In a second tube put 5 cc. of working standard uric acid solution and 5 cc. of water. (3) From a burette add to each 4 cc. of 5% sodium cyanide solution (poison). (4) From a burette add to each 1 cc. of uric acid reagent (poison). (5) Mix, and put in boiling water bath 3 minutes. (5) Cool 3 minutes in a basin of water. (6) Read at once in colorimeter. Calculation:

$$\frac{\text{Standard}}{\text{Unknown}} \times 4 = \text{mg.}$$
 uric acid per 100 cc. blood.

The use of an excess of oxalate in collecting the blood may cause clouding of the solution. This may sometimes be avoided by repeating the test, diluting both the filtrate and standard solution with ro cc. of water instead of 5 cc.

Solutions. Sodium cyanide solution.—Prepare a 5% solution, adding 2.0 cc. ammonia to a liter of solution. This should not be allowed to stand in the burette, but in a stoppered bottle. It must be filtered before use, and made up fresh every two months.

Uric acid reagent.—In a liter flask put 100 Gm. sodium tungstate, and dissolve in 600 cc. water. Add successively 50 Gm. arsenic pentoxide, 25 cc. of 85% phosphoric acid, and 20 cc. concentrated HCl. Boil 20 minutes, cool, and dilute to 1 liter. It keeps indefinitely.

Uric acid standard solution (Folin).—(Folin's solution is more stable than Benedict's, and preferable where only occasional tests are made.)

Stock solution.—(1) Weigh out I Gm. of pure uric acid, and transfer to a liter volumetric flask. (2) Dissolve o.6 Gm. of lithium carbonate in 120 cc. of hot water, and filter. (3) Add 60 cc. water to this filtrate, heat to 65°C., heat the flask similarly, and add solution, washing in with it traces of uric acid left on the weighing glass and funnel.
(4) Shake till dissolved (5 to 15 minutes). (5) Cool and dilute to 800 cc. with water.

(4) Shake till dissolved (5 to 15 minutes). (5) Cool and dilute to 800 cc. with water.

(6) Add 10 cc. clear formalin (saturated solution). Mix. (7) Add 15 cc. strong sulphuric acid to 100 cc. water, cool, and add to the flask. (8) Dilute to the mark and mix. If stored in small, filled, tightly stoppered bottles it keeps for 6 to 12 months.

Working solution.—(1) In a 250 cc. flask half full of water add just 1 cc. of stock solution. (2) Add 1 cc.  $\frac{2}{3}$ :N H<sub>2</sub>SO<sub>4</sub> (or 80 cc. N/12 acid). (3) Add 1 cc. formalin.

(4) Dilute to the mark and mix. This keeps for several weeks.

Significance.—Normal blood contains from 2 to 4 mg. % of uric acid. The uric acid in the blood and tissue fluids is derived in part from the purins of the food (exogenous), and the balance (largely at least) from the disintegration of nucleic acid from the body tissues (endogenous). It is disposed of in part by excretion in the urine, and to a large but variable extent by oxidation in the body, roughly about 50%. The blood uric acid content is thus the resultant of at least 4 variable factors. A diet high in purins has little effect on the blood uric acid in normal individuals, but increases it somewhat in gout and in nephritis. A diet high in purin-free protein, or in carbohydrate increases uric acid excretion, and lowers blood uric acid. On the other hand, a diet high in fut has the opposite effect and may raise blood uric acid to 10 or 12 mg. %. Starvation has a similar effect. In gout there is usually a moderate increase, from 4 to 10 mg. %. The figures are not always definitely abnormal, and often can be reduced by dietary treatment or by the administration of salicylates or cinchophen. In nephritis there is often a moderate increase, even in cases with only slight impairment of renal function. This is often seen in cases of arteriolosclerosis and essential hypertension. It may rise to 10 or even 20 mg. % in uraemia. However, there is no close parallelism between the height of the blood uric acid and the degree of renal insufficiency, and in general this increase is so uncertain and variable that it "has been abandoned" (by Peters and Van Slyke) "as an aid in diagnosing and classifying the nephritides." High figures have been reported frequently in the toxaemias of pregnancy, in leukaemia, polycythaemia, pneumonia, chronic passive congestion, and occasionally in a variety of other conditions. In general a normal blood uric acid is more valuable in excluding gout, then a high figure, in proving it. A moderately high figure not otherwise explained is suggestive of early renal disease, but a normal figure does not exclude it. It is usually not increased in intestinal obstruction and similar conditions, even though the N.P.N. is high. Uric acid estimations, therefore, have relatively little practical value.

#### Glucasa

Tests for the quantitative determination of sugar in the blood are based on its property of reducing cupric salts in alkaline solution. There are other reducing substances present in the blood, particularly in the cells, which contribute to the reaction,

and make the blood sugar figures erroneously high. In the Folin-Wu and other early methods the error ranged from 20 to 30 mg. %. The new methods of Benedict, and of Folin-Wu (which follows), reduce this to about 10 or 12 mg. %, and consequently give blood sugar readings about 20 mg. % lower than the older methods, on which published statistics and discussions in the literature are based. This fact must be remembered in interpreting the results of the newer and more accurate procedures.

Determinations may be made either on venous blood, as is the usual custom in this country, or on "capillary" (finger tip) blood, as has been customary in Europe. Equally precise determinations can be made with blood from either source, but except in the fasting state they do not give identical results, venous blood containing less sugar than capillary blood, which is practically arterial blood. This is discussed in detail in connection with glucose tolerance tests.

Folin-Wu improved method, applicable to venous blood.—(1) In a Folin sugar tube put 2 cc. of blood filtrate. (2) In a second tube put 2 cc. of single glucose standard solution, and (3) in a third tube put 2 cc. of double standard. (4) To each add 2 cc. of freshly mixed copper tartrate solution. (5) Heat in boiling water bath for 8 minutes. (6) Cool in water. (7) Add 4 cc. of acid molybdate reagent. (8) After 1 minute, dilute to volume with diluted reagent. (9) Mix and compare in colorimeter.

Calculation:

 $\frac{20}{\text{Reading}} \times 100 = \text{mg. sugar per 100 cc.}$  (Multiply by 200 if the double

standard was used.)

Standard solution of glucose (stock).—In a volumetric flask dissolve r Gm. of anhydrous glucose (Burcau of Standards) in 100 cc. saturated benzoic acid solution. r cc. = 10 mg. It keeps for years.

Intermediate standard.—Dilute 100 cc. of stock solution to 500 cc. with benzoic acid solution. This also keeps indefinitely. r cc. = 2 mg.

Double working standard.—Freshly dilute 10 cc. of the preceding to 100 cc. with half saturated benzoic acid solution. 1 cc. = 0.2 mg.

Single working standard.—Dilute 5 cc. of the intermediate standard solution to 100 cc., similarly. 1 cc. = 0.1 mg.

Copper sulphate solution.—Dissolve 25 Gm. crystallized copper sulphate in some water in a 500 cc. volumetric flask, add 5 drops of concentrated sulphuric acid, dilute to volume.

Alkaline tartrate solution.—In a 1 liter volumetric flask put 35 Gm. of anhydrous sodium carbonate, add 200 cc. of water, and shake until dissolved. Add 13 Gm. sodium tartrate (Merck's highest purity) and 11 Gm. of sodium bicarbonate. Add about 600 cc. more water, shake until clear, dilute to volume, and mix.

Alkaline copper tartrate solution.—Prepare daily by adding 9 volumes of the alkaline tartrate solution to 1 volume of the copper sulphate solution.

Acid molybdate reagent.—(1) In a 1 liter volumetric flask put 300 Gm. of sodium molybdate, add about 800 cc. of water, and shake until dissolved. Dilute to the mark, mix, transfer to a bottle, and add 0.3 cc. of liquid bromine, shake, and let stand. (2) In a large flask put 500 cc. of the clear supernatant fluid, and add, while stirring, 225 cc. of 85% phosphoric acid. Then add 150 cc. of cool 25% (by volume) sulphuric acid, and let stand over night. Pass an air current through the solution to remove the bromine.

Add 75 cc. of 99% acetic acid. Mix, and dilute to  $\tau$  liter in a volumetric flask. This keeps indefinitely.

Diluent.—Add I volume of this reagent to 4 volumes of water.

Folin's Ferricyanide Micro-method.—(1) In a centrifuge tube put 4 cc. of sulphate-tungstate solution. (2) Fill to the mark a pipette accurately graduate to contain 0.1 cc. with blood from the finger tip (or vein), wipe off any blood that may adhere to the outside of the pipette, and discharge the blood into the tungstate solution. Rinse out pipette with solution in the tube. Mix. (3) After 15 minutes or longer add 1 cc. of sulphuric acid-sulphate solution, mix, and centrifuge for 5 minutes. (4) Put 2 cc. of the clear solution and 2 cc. of water in a test tube graduated at 25 cc. In a similar tube put 4 cc. of standard glucose solution. (5) To each add 1 cc. of 0.4% potassium ferricyanide solution (2 cc. if a high blood sugar is expected), and (6) 1 cc. of cyanide-carbonate solution. (7) Heat in boiling water bath 8 minutes; cool in running water 2 minutes. (8) Add 5 cc. of ferric iron solution and mix. (9) After 2 minutes dilute to the mark (adding 3 drops of alcohol to get rid of foam as the mark is approached, if necessary) and mix. (10) Put standard solution in both colorimeter cups, set both at 20, insert yellow light filter, and adjust light until they are exactly alike. (11) Read unknown in the colorimeter.

Calculation:

This method gives proportionately accurate readings with the unknown solution within the range of 5 mm. to 40 mm. provided the light and color filter are properly adjusted. It gives slightly higher readings than the preceding method. Without the light filter it is necessary to prepare numerous standard solution tubes of varying concentration, and use one that closely approximates the color of the unknown solution.

Sulphate tungstate solution.—In a 500 cc. volumetric flask, put 10 Gm. anhydrous sodium sulphate, and 15 cc. of 10% sodium tungstate solution. Dissolve in water, fill to volume and mix.

Sulphuric acid.—In a 100 cc. volumetric flask put 12 cc. of 23 N sulphuric acid, and 2 Gm. of anhydrous sodium sulphate, dissolve, fill to volume with water, and mix.

Potassium ferricyanide solution.—Dissolve 2 Gm. of pure salt in water, and dilute to 500 cc. Keep in the dark in brown bottles.

Cyanide-carbonate solution.—Put 8 Gm. anhydrous sodium carbonate in a 500 cc. flask, add 50 cc. water, shake till dissolved. Add 150 cc. of freshly prepared 1% sodium cyanide solution. Fill to the mark, mix, filter if necessary.

Ferric iron solution.—Fill a 1 liter graduate with water. Put 20 Gm. of gum ghatti in a tea ball, and suspend in the top of this water for 18 hours. Filter through a double layer of clean towel. Add to the extract a solution containing 5 Gm. anhydrous ferric sulphate dissolved in 75 cc. of 85% phosphoric acid and 100 cc. water. To remove reducing substances in the gum ghatti, add a little at a time about 15 cc. of 1% KMnO4 solution, until the pinkish tint persists for some minutes. Turbidity if present will clear if kept at 37°C. for a few days.

Standard glucose solution contains 0.01 mg. per 1 cc. Dilute to 200 cc., 1cc. of the intermediate standard (B) prepared for the previous method, using 25 cc. of 0.2% benzoic acid and water to the mark. Keep under toluol. Renew frequently.

Yellow light filter.—Dissolve 5 Gm. picric acid in 100 cc. methyl alcohol, and add 5 cc. of 10% sodium hydroxide. Secure 10 pieces of heavy filter paper such as Schleicher and Schull No. 604, large enough to cover the opening of the colorimeter lamp, put on a mat of newspapers and pour over them the solution of picric acid until the solution filters through at the bottom, and spreads out 2 cm. from the filter papers at each side. Dry. Pour over them an excess of a 5% solution of paraffin in benzine, and dry. Mount in some frame which can be inserted conveniently between the colorimeter lamp and the instrument. A powerful light is necessary (150 Watt Mazda) but the daylite glass can be removed. To test, put water in one cup, and 0.2% potassium ferricyanide solution in the other. Set both at 20. If adequate, the two can easily be made equal.

A suitable glass filter can be purchased (Spencer Lens Co., Buffalo, N. Y.).

Significance.—The normal fasting blood sugar (either in venous or capillary blood) ranges from 70 to 120 mg. % by the older methods, and from 60 to 100 mg. % by the methods above given. In elderly individuals the normal maximum figure is from 10 to 20 mg. higher. It may be reduced by starvation, particularly in children, even to 40 mg. %. Lower figures are also seen in normal individuals 4 to 6 hours after a meal. A transient rise in blood sugar may follow exposure to cold or extreme heat, painful stimuli, emotional disturbances of any sort, asphyxia, and the administration of various drugs, such as general anesthetics, morphia, amytal, caffeine, quinine, epinephrin and ephedrin.

A persistent increase in blood sugar is most often seen in diabetes, the fasting sugar ranging in untreated individuals from 100 to 300 mg. % in the milder cases, and up to 1200 mg. % in the most severe. A pathological increase may also occur in hyperthyroidism and hyperpituitarism, some adrenal tumors, in some acute infections, particularly pyodermias, in chronic diseases of the liver and bile ducts, in some malignant neoplasms. in various intracranial lesions, and in some cases of essential hypertension with good renal function and of chronic nephritis with hypertension, especially with nitrogen retention.

Pathologically low figures, from 60 to 30 mg. %, have been reported in cases of neoplasms of the pancreas involving the islands of Langerhans. accompanied by excessive insulin production, and characterized clinically by severe hypoglycaemic reactions. A similar milder clinical disturbance with a lesser reduction in blood sugar is not rare in individuals without gross lesions of the pancreas, and has been attributed to functional overactivity of the islands. The blood sugar may be low in cases of hypopituitarism and of Addison's disease.

Blood sugar estimations are of greatest practical value in the recognition of diabetes and in controlling its treatment. Before the diagnosis of

diabetes can be established, other causes of hyperglycaemia must be excluded.

# Carbohydrate Metabolism

The carbohydrates of the food are absorbed chiefly as hexoses; glucose, fructose and galactose. Small amounts of other sugars are also absorbed. Except for maltose, the disaccharides (sucrose, lactose, etc.) can not be utilized by the tissues, and if absorbed they are totally excreted in the prine.

After absorption these sugars are carried by the portal circulation to the liver where a large proportion is abstracted and converted into glycogen. A high blood sugar seems to be the immediate stimulus for this conversion. In addition to these sugars certain simpler substances can be converted into glycogen in the liver, such as the trioses, dihydroxiacetone and glycerin (which constitutes 10% of the fat), and d-lactic acid. Amino-acids representing about 54% of the protein catabolized are de-aminized and converted into glycogen in the liver.

Such glucose and maltose and possibly fructose as escape the liver (but probably not the other substances) may be oxidized directly in the tissues, or they may be taken up by the muscles and stored as muscle glycogen. The energy for muscular contraction is supplied by the degradation of muscle glycogen (anaerobically) into d-lactic acid. The latter is in large part converted into glycogen in the liver (and perhaps to some extent in the muscle), a portion being oxidized to supply the energy necessary for the conversion. (Insulin appears to be necessary for this process.)

Maintenance of a normal concentration of glucose in the blood and tissues depends upon the breakdown of glycogen into glucose and its orderly release by the liver. A low blood sugar supplies the stimulus for this glycogenolysis. The glycogen of muscle does not directly give rise to glucose, although it may contribute indirectly, since the lactic acid formed from it is converted into glycogen in the liver and is then available for glucose formation. However even in the diabetic it is so utilized only to a limited extent.

These processes are controlled by the interaction of various hormones of which insulin and epinephrin have been most carefully studied and are probably of major importance. The anterior pituitary, the thyroid and probably the adrenal cortex participate.

The major functions of *insulin* are: (1) It inhibits the conversion of glycogen into glucose by the liver. (2) It stimulates the formation of

glycogen in the liver. (3) It stimulates the conversion of glucose into muscle glycogen. (4) It increases the oxidation of glucose or its decomposition products in the tissues. As a result of the last two functions, a hypoglycaemia tends to develop, and this may lead to a secondary glycogenolysis and depletion of liver glycogen (even in the absence of the adrenals). It does not directly influence the breakdown of muscle glycogen into lactic acid.

The principal effects of *epinephrin* on carbohydrate metabolism are: (1) It stimulates glycogenolysis and raises the blood sugar level. (2) It increases the breakdown of muscle glycogen into lactic acid. (3) It appears to lessen the oxidation of glucose in the tissues. It does not inhibit the production of glycogen in the liver. On the contrary, the lactic acid liberated in the muscles is rapidly converted into glycogen in the liver, and this may bring about an increase in liver glycogen as a late result. In most respects, therefore, insulin and epinephrin are direct antagonists.

The "diabetogenic" hormone of the anterior pituitary stimulates glycogenolysis, probably by a direct action on the liver. Its precise functions and mode of action have not yet been determined. Hypophysectomy results in hypoglycaemia, markedly ameliorates the diabetes in a depancreatized animal, and increases the sensitiveness to insulin. This anterior pituitary hormone is thus an antagonist of insulin. Thyroxin increases the response to epinephrin. The adrenal cortex also appears to be an antagonist of insulin. Hypoglycaemia and an increased sensitiveness to insulin are met with in adrenalectomized animals and in severe Addison's disease.

In the *diabetic* these functions of insulin in large measure are lost. There is a gross impairment of the capacity to form and store glycogen and to oxidize glucose. As a secondary result of the latter, fats are incompletely oxidized, and lipaemia, ketosis and acidosis result. It is not yet certain which of these disturbances is primary. It has been commonly assumed that the primary defect in diabetes is the loss of the power to oxidize glucose, and that the other disturbances are secondary to this. This view is based in part on the low respiratory quotient (R.Q.) which is believed to indicate that only fat is being oxidized, and on the fact that the R.Q. is elevated by insulin. However, the R.Q. may be influenced by other factors than the composition of the food mixture which is being oxidized, which are difficult to control experimentally. There is considerable experimental evidence that the totally diabetic animal retains to some extent the capacity to oxidize glucose and to form glycogen.

Such facts have led some to believe that the primary disturbance is unrestricted gluconeogenesis, an overproduction of glucose, and that deficient oxidizing power is a secondary result.

There is also controversy as to whether all cases of human diabetes are due to primary disease of the pancreas. Significant lesions of this organ are not uniformly found at autopsy. Some cases of diabetes are unusually refractory to the therapeutic action of insulin. Further study may possibly show that some cases are due to overactivity of the anterior pituitary. It seems improbable that true diabetes results from a primary disturbance of the thyroid or adrenal (or the liver) although hyperglycaemia and glycosuria occur in some diseases of these organs.

The glucose tolerance test was devised as a clinical test of the integrity of the mechanism controlling the assimilation of glucose. The normal assimilation limit of glucose has been determined precisely in animals by continuous intravenous injections of glucose. If the rate of injection is o.8 Gm. per kilo per hour or less, no glucose is excreted in the urine. If the rate of injection is from o.9 to 2.0 Gm. per kilo per hour, some glucose is excreted, the amount increasing as the rate of injection rises but not exceeding one tenth of the amount injected. If more than 2 Gm. per kilo per hour is injected, a large proportion of the excess over 2 Gms. is excreted. Since the maximum rate of absorption from the intestine never exceeds 1.8 Gm. per kilo per hour, no notable loss of glucose in the urine is to be anticipated after its administration by mouth in any amount to a normal individual.

When a normal individual takes a substantial amount of glucose (50 Gm. or more) by mouth, the blood sugar quickly rises. As it rises, it stimulates the process of conversion of the excess of glucose into glycogen and its storage in the liver and muscles. This process goes on with increasing rapidity, and soon equals and exceeds the rate of absorption from the intestine. As a result the blood sugar begins to fall sharply and abruptly (in 20 to 40 minutes), as a rule before the rising renal threshold for glucose has been exceeded (see p. 721). The storage process continues until the blood sugar (after the 3rd or 4th hour) has fallen well below the fasting level.

To map out the course of the blood sugar precisely and particularly to determine the peak of the curve (which is maintained only for a few minutes) and the threshold level, if sugar is excreted, it is necessary to obtain blood (and urine) specimens at frequent intervals, at least every 10 minutes. For these purposes finger tip blood is essential, because during its circulation through the muscles of the arm the blood loses a large and variable amount of glucose. The venous blood sugar at the peak (140 to 160 mg.) is from 30 to 70 mg. lower than the capillary blood sugar (170 to 220 mg.). As the blood sugar falls, this difference diminishes, but the venous blood sugar remains the lower until the fasting level is regained several hours later. It is manifestly the capillary blood sugar level which determines excretion of sugar by the kidney, and which is the more significant figure.

In normal individuals increasing the amount of glucose administered above 50 Gm. (to 100 or even 200 Gm.) does not, as one might expect, increase the height of the peak of the curve. Increasing the amount of glucose administered does not accelerate the rate of absorption. The storage mechanism, once in operation, can keep pace with the maximum rate of absorption. However, such large amounts prolong the period of absorption and delay the fall in blood sugar to the fasting level.

In diabetes the response is entirely different. The mechanism for storage of carbohydrate (as glycogen) is disturbed, as well as that for its oxidation. It is removed from the blood very slowly, and as a result the blood sugar rises rapidly to a much higher level, well above the rising threshold; the peak is flatter and more protracted; and the return to normal is much delayed, so that the blood sugar is still high 3 and 4 hours after the glucose administration. In diabetes the height of the peak does vary with the amount of glucose given, and there is less difference between the venous and capillary blood sugar. In milder cases every transition may be met with from a normal response to an outspoken diabetic curve.

The diabetic response, then, differs from the normal in two major respects. The peak is higher and the return to normal is much delayed. As a routine clinical procedure it is manifestly impracticable to make a sufficient number of observations to determine the peak precisely, and chief reliance must be placed on the delay in the return to normal. This varies materially with the dose of glucose administered. The usual custom has been to give 100 Gm. of glucose, or 1.75 Gm. per kilo; but this quantity may well be somewhat injurious to an outspoken diabetic, and in normal cases it tends to obscure rather than emphasize the differences between a normal and a pathological response. The optimum dosage is lower, from 50 to 75 Gm., or preferably 1.0 Gm. per kilo. The smaller quantities are also less likely to cause nausea. The only disadvantage is that the current standards of normal are based on tests with the larger quantities, and the criteria for tests with smaller quantities must be somewhat more strict. After 1.0 Gm. per kilo the glucose in capillary blood returns to normal after 2 hours (average) to a maximum of 2.5 hours or, in elderly individuals, 3 hours. The venous blood sugar reaches normal 30 to 60 minutes sooner. Increasing the dose to 1.5 or 1.75 Gm. per kilo prolongs the time about 1 hour. For determining the duration of the curve examinations of venous blood are satisfactory.

Procedure.—A glucose tolerance test should be carried out whenever diabetes is suspected. It is usually inadvisable in frank cases. The test should be carried out in the morning and the patient should have fasted since supper. Previously he should have been on an unrestricted diet. He should rest quietly during the test, and emotional disturbance must be avoided. (1) The bladder is emptied, the urine being saved, and blood is drawn for sugar determination (either from the vein or preferably from the finger if the peak or threshold is to be determined). (2) The patient at once drinks a solution of 1 Gm. of glucose per kilo of body weight dissolved in about 250 cc. of water. (3) Collect urine and blood at 30 minutes, 1 hour, 2 hours, and 3 hours after taking the glucose (the 2 hour specimen is the most important). (4) Make quantitative determinations of glucose on all the specimens. Nothing except water may be taken until the test is completed.

Interpretation.—A normal individual shows a fasting blood sugar usually under 100 mg. %. The maximum figure observed (usually at the

½ hour interval) does not exceed 180 mg. % in venous blood (220 mg. % in capillary blood) and may be very much less, since the actual peak is only occasionally observed when specimens are taken so infrequently. The figure drops substantially to the fasting level after 1½ to 2 hours in venous blood, and 2 to 2½ hours in capillary blood, and well below this after 3 hours (often to 60 mg. % in venous blood.) John regards 120 mg. % 3 hours after 100 Gm. of glucose as conclusively pathological.

A normal curve practically excludes diabetes. The fasting blood sugar may not be elevated in mild diabetes, particularly in young individuals. The amount of sugar excreted in the urine is of minor importance. No stress can be laid on slight deviations from the normal. The test is disturbed by variations in the rate of absorption of glucosc. Particularly if it causes nausea, gastric motility may be altered so as to delay absorption and give a more protracted curve with a lower peak. A prolonged curve indicating impaired tolerance is found not only in diabetes, but in other conditions in which a high fasting blood sugar occurs, particularly in overactivity of the thyroid, pituitary, and adrenal, and in some cases of obesity and of hypertension and nephritis. In hyperthyroidism it is common to observe a high peak, with relatively little delay in the fall to normal.

In cases of hyperinsulinism the peak of the curve is lower than normal and the blood sugar falls to pathologically low figures (40 to 60 mg.) at the fourth, fifth or sixth hour after administration of glucose. In sprue and idiopathic steatorrhoea (but not in pernicious anaemia or pellagra) the curve is typically flat with a low peak.

Renal glycosuria ("renal diabetes").—This is a condition characterized by the excretion of glucose in the urine of individuals who show a normal (or low) blood sugar, and no disturbance in the capacity to oxidize and utilize carbohydrate (low renal threshold). The glycosuria is independent of the diet, and may occur during a fast. It may be transient or continuous. It is usually slight or moderate, but it may be marked. Glucose tolerance tests show normal or shortened curves. The condition is a constitutional anomaly which does not affect the health, shorten life, or predispose to diabetes. Carbohydrates should not be restricted. In its milder forms it is fairly common. Precise differentiation from diabetes is most important, and may require a considerable period of observation.

# Chlorides

Determinations of chloride in whole blood are of less value than in serum or plasma because of the fact that the concentration of chloride in the cells is only about half that in the plasma, and differences in chloride content of whole blood due to differences in the volume of the cells associated with anaemia or polycythaemia overshadow those commonly met

with in disease. However, Cl passes from the cells to the plasma when blood is exposed to the air, because of the escape of CO<sub>2</sub> from the blood. To avoid this error it is, therefore, preferable to collect the blood under oil and to remove the serum as promptly as possible without exposure to the air, and this is essential for research accuracy.

Procedure (Van Slyke and Sendroy).—(1) Put in a large pyrex test tube 1 cc. of plasma or serum (or whole blood) which has been collected under oil and not exposed to the air until after separation from the cells. (2) Add slowly with constant stirring 3 cc. of the silver nitrate-nitric acid reagent. (3) Cover mouth of the tube with a watch glass, and put in a steam bath or boiling water bath until the fluid over the AgCl precipitate is clear and light yellow in color. (Requires 1 to 2 hours; if necessary decolorization can be completed by adding a drop at a time a few drops of saturated KMnO<sub>4</sub> solution.) (4) Cool to room temperature or lower. (5) Add 6 cc. of 5% ferric alum solution, or 6 cc. water and 0.3 Gm. of powdered ferric alum. (6) Titrate excess of silver nitrate with 0.02 N sulphocyanate solution until a pink color is obtained that persists 15 seconds.

Calculation:

 $71 \times (7.54 - \text{number of cc. of sulphocyanate solution used}) = \text{mg. Cl per}$  100 cc. plasma. (To express as NaCl, substitute 117 for 71.)

Silver nitrate-nitric acid reagent.—Dissolve 8.495 Gm. fused silver nitrate in a minimum amount of water, and dilute to r liter with concentrated nitric acid. (It keeps indefinitely.)

0.02 N sulphocyanate solution.—Dissolve 1.5 Gm. ammonium sulphocyanate in about 000 cc. of water. Check the strength of the solution by titrating against 3 cc. of the silver nitrate-nitric acid solution and adjust so that just 7.54 cc. are required. This solution weakens on standing and must be rechecked frequently.

Whitehorn's Method.—Determination of the chlorides in whole blood can be made from the Folin-Wu tungstic acid filtrate. (r) To 10 cc. of filtrate add 5 cc. of AgNO<sub>3</sub> solution (containing 4.791 Gm. per liter, r cc. = r mg. Cl), mix, and add 5 cc. concentrated nitric acid. After 10 minutes add 0.3 Gm. of powdered ferric alum, and titrate with standard NH<sub>4</sub>CNS solution. This is prepared by dissolving 2.3 Gm. ammonium sulphocyanate in a liter of water, and adjusting by titration and dilution so that 5.04 cc. corresponds exactly to 5.0 cc. of the silver nitrate solution. The end point is a salmonred tint that persists for 15 seconds in spite of stirring.

Calculation:

 $(5 - \text{No. of cc. of sulphocyanate solution used}) \times \text{roo} = \text{mg. Cl per}$ 

Significance.—The normal Cl content of whole blood is 273 to 303 mg. per 100 cc. and of plasma 352 to 383 mg. %, corresponding to 560 to 630 mg. % of NaCl, or 99 to 108 millimoles (mM) per liter. The extremes of normal are from 95 to 110 mM, and fluctuations in disease usually do not

exceed these limits materially. Hence great precision in technique is required to get determinations of any value.

One of the major functions of NaCl is to maintain the normal osmotic pressure in the body fluids. Some unknown mechanism in which the kidney must play the major rôle keeps the electrolyte content of the plasma nearly constant. Urine excretion of chloride practically stops when the plasma chloride falls below 96 mM. If chloride is ingested faster than it can be excreted, it does not accumulate in the plasma but passes (as NaCl and water) into the intercellular tissue spaces and produces oedema. In chronic passive congestion, "nephrosis" and active chronic nephritis with oedema this tendency is exaggerated, because the mechanism for the excretion of water and salt is impaired in these conditions.

Transient rises in plasma chloride within or slightly in excess of these limits may occur in cases on a salt-rich diet; with restriction of fluid; with hyperventilation which reduces CO<sub>2</sub> tension and causes a reciprocal rise in Cl; in some cases of "nephrosis" or nephritis with oedema (but 'surprisingly inconstant and slight); after the administration of acidif; sying chloride diuretics (NH<sub>4</sub>Cl, CaCl<sub>2</sub>); and in some cases of advanced chi ronic nephritis without oedema, with a fairly liberal salt intake.

Reductions of similar magnitude may occur in cases on a salt po—or diet; after excessive water drinking; in starvation; in emphysema, and bother conditions associated with inadequate pulmonary ventilation and the dollar conditions; in venous stasis; with extensive burns; and in various the acute infections, particularly pneumonia, in which lowering may be excessive, to 80 mM.

More marked reduction of chloride may occur in severe diabetic ket uosis, in which plasma Cl is replaced by organic acids and excreted in the ureline. It also follows excessive loss of chloride through other channels than the kidney, as after profuse sweating resulting from heavy labor at high temperatures (heat exhaustion, stoker's cramps); or persistent diarrhoed, as in cholera; and in cases with duodenal fistula. Maximum depletion, even to half the normal figures, follows intractable vomiting, particularly when associated with pyloric or high intestinal obstruction, but also in toxaemic vomiting of pregnancy, mercuric chloride poisoning, etc.

If HCl is vomited, an alkalosis from loss of acid is also produced, with a high bicarbonate content in the plasma. If achlorhydria is present, there is no alkalosis, but there is an equal loss of plasma electrolytes, and lowering of both sodium and chloride. Although the depletion of base (chiefly sodium) is probably of primary importance, determinations of sodium are time-consuming and impracticable as a routine procedure.

Peters has pointed out that the sum of the serum chloride and serum bicarbonate expressed in mM closely approximates the total sodium of the serum, and (usually) is practically a measure of the blood electrolytes.

In these conditions the giving of water is harmful, since the body is unable to retain water without sufficient salt to make it isotonic with the body fluids. Neither will salt be retained or absorbed unless adequate water is also given. The physiological activities are so adjusted as to resist or minimize any change in osmotic pressure, and failure to do so is speedily fatal. However, parenteral injections of physiological or preferably hypertonic salt solution relieve symptoms and prolong life.

In essential hypertension and in chronic nephritis without oedema there is no change in plasma chloride or in chloride excretion except when renal insufficiency has become marked. Then the kidney loses its power to control chloride excretion. It is unable to concentrate NaCl normally in the presence of an excess, or to hold it back when plasma chloride is low. Hence either high or low figures may be met with, depending largely upon the NaCl intake. In uraemia plasma chloride is more often reduced, sometimes markedly so.

Plasma chloride determinations are of especial value in detecting and measuring the more marked degrees of chloride depletion in the conditions which have been enumerated, and in guiding their treatment with NaCl injections.

# Calcium and Phosphorus

**Calcium.**—Determinations are made on the *scrum*. There is practically no calcium in the red cells.

Clark-Collip modification of Kramer-Tisdall method.—(1) In a 15 cc. graduated centrifuge tube with a narrow tip (having an outside diameter of 6 to 7 mm. at the 0.1 cc. mark) put 2 cc. of clear serum. (2) Add 2 cc. of water and 1 cc. of 4% ammonium oxalate solution. (3) Mix thoroughly, and let stand 30 minutes or longer. (4) Mix, and centrifuge at high speed for 5 minutes. (5) Decant the supernatant fluid carefully, taking care not to agitate the precipitate, put the inverted tube on a pad of filter paper, and drain 5 minutes. (6) Wipe the mouth of the tube dry, wash down the walls of the tube with 3 cc. of dilute ammonia solution, and resuspend the sediment. (7) Centrifuge and drain as described. If carefully done, the slight loss of sediment is compensated for by the trace of ammonium oxalate remaining in the precipitate. (8) Add 2 cc. of normal sulphuric acid so as to break up and dissolve the precipitate, and heat in a boiling water bath for a minute. (9) Keep the solution at 70°C. or higher, and titrate with a o.or N solution of potassium permanganate using an accurate 5 cc. microburette until a definite pink color lasting one minute is obtained. (10) Check the strength of the permanganate solution by titration with freshly prepared 0.01 N solution of sodium oxalate acidified with a few drops of strong sulphuric acid, and correct the reading. If, e.g., 10 cc. of sodium oxalate solution (0.01 N) requires 11 cc. of 0.01 N permanganate solution to give a pink color, the reading observed under (9) should be multiplied by 1911.

Calculation:

1 cc. of 0.01 N KMnO4 solution = 0.2 mg. Calcium.

Burette reading 
$$\times \left(0.2 \times \frac{100}{2}\right)$$
 = mg. Calcium per 100 cc. serum.

Reagents.—(1) Ammonium oxalate solution: dissolve 4 Gm. in 100 cc. of water.

- (2) Dilute 2 cc. of strong ammonia solution to 1 liter with water.
- (3) Dilute 28 cc. of concentrated sulphuric acid to 1 liter with water.
- (4) N/10 potassium permanganate solution (Halverson and Bergein).—Dissolve 4 Gm. pure KMnO4 in one liter of distilled water, in a Florence flask. Put funnel in the mouth of the flask, cover with a watch glass, heat barely to boiling, and digest for several hours. Cool, let stand over night, and filter through glass wool, or through ignited asbestos in a Buchner funnel, by suction. Store in brown glass-stoppered bottles in a dark place. After a preliminary period of relatively rapid weakening it keeps almost unchanged for many months. Its strength must be checked by titration with N/10 sodium oxalate or oxalic acid solution and adjusted. (Theoretically it should contain 3.158 Gm. per liter.)
- (5) N/100 potassium permanganate solution.—Dilute 10 cc. of the N/10 solution to 100 cc. with distilled water. This weakens rapidly, and its strength must be checked daily. (It may not be filtered through paper. If turbid, centrifuge.)
- (6) N/10 sodium oxalate solution.—Dissolve 6.7 Gm. of the Sørensen salt in 1 liter of water containing 5 cc. of concentrated H<sub>2</sub>SO<sub>4</sub>. It keeps indefinitely.
- (7) N/100 sodium oxalate solution.—Dilute 10 cc. of (6) to 100 cc. with water. It should be freshly prepared each week. N/100 oxalic acid solution may be used.

The normal figures are 9 to 11.5 mg. % for adults, and 10 to 11.5 mg. % for children.

Significance.—Calcium occurs in serum in two forms. One part, (3 to 5 mg. %), which appears to be physiologically inactive, is kept in solution by a combination with plasma protein, and is diminished in any condition in which plasma protein is diminished. The balance, which is the physiologically active calcium, behaves as if it existed in plasma as Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> in saturated solution in contact with undissolved Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> (in the bones). Anything which causes a change in the amount of either calcium or inorganic phosphorus in the plasma causes a reciprocal change in the other, by abstracting it from, or depositing it in the bones, which act as a storehouse for these elements. Thus, administration of soluble calcium salts (CaCl2) tends to raise blood Ca, and indirectly lower blood P, while administration of soluble acid phosphates tends to raise blood P, and lower Ca. To interpret the significance of changes in blood calcium, it is therefore necessary to know the amount of both total plasma protein and inorganic P present. These changes are discussed more fully in conjunction with P in the following pages.

Inorganic phosphorus (Benedict and Theis).—This method is suitable only for clear serum or plasma free from hæmoglobin, not whole blood. (For research purposes the method of Fiske and Subbarow is recommended, but the solutions required are less stable.) (1) In a test tube put 2 cc. of clear serum or plasma, (2) add 4 cc. of water, and (3) add 4 cc. of 20% trichloracetic acid. (4) Shake; let stand 10 minutes or longer; shake again, and filter through a small paper. (5) In a 10 cc. graduate put 3 cc. of filtrate (with a volumetric pipette) and 2 cc of water. (6) In another graduate put 5 cc. of standard phosphate solution containing 0.025 mg. of P. (7) To each add 3 cc. of 16% H<sub>2</sub>SO<sub>4</sub> (by weight), (8) 1 cc. of freshly diluted molybdic acid reagent, and (9) 1 cc. of hydroquinone-bisulphite reagent. (10) Pour into test tubes and heat in a boiling water bath for 10 minutes. (11) Cool, and read in colorimeter.

Calculation:

$$\frac{\text{Stand.}}{\text{Unkn.}} \times \text{0.025 mg.} \times \frac{5 \text{ (dilution of blood)} \times 100}{3} = \text{mg. } \% \text{ P.}$$

If standard is set at 20, simplified,

$$\frac{250}{\text{Reading}} = \text{mg. } \% \text{ P.}$$

**Reagents.** Phosphorus standard solution (stock).—Dissolve 0.4394 Gm. of dry monobasic potassium phosphate in one liter of water. Preserve with a few drops of chloroform. 10 cc. = 1 mg.

Phosphorus standard solution (working standard).—Dilute 5 cc. of the stock solution with water, to 100 cc. Preserve with chloroform. 5 cc. = 0.025 mg.

Molybdic acid reagent.—To 20 Gm. of pure molybdic acid add 25 cc. of 20% sodium hydroxide and warm gently until dissolved. Cool and dilute to 250 cc. Filter if necessary.

Molybdic acid, working reagent.—Dilute (freshly each day) with an equal part of water, a little of the stock solution.

Hydroquinone-bisulphite reagent.—Dissolve 15 Gm. of sodium bisulphite and 0.5 Gm. hydroquinone in 100 cc. of water.

Sulphuric acid, 16% by weight.

The normal figures for inorganic P in serum or plasma in adults range from 2.5 to 5.0, usually about 3.5 mg. per 100 cc., and in children from 4 to 7, usually about 5 mg. %. The phosphorus in organic combination in the blood, particularly in the red cells, is far greater than the inorganic phosphorus, but thus far no information of practical clinical value has been obtained from its estimation. This method, if applied to whole blood, includes a large amount of acid-soluble organic P from the cells, and gives figures which are far higher than for serum and are at present meaningless. The figures which follow refer to inorganic serum P.

Significance of Blood Calcium and Inorganic Phosphorus.—The amount of calcium phosphate dissolved in plasma is about 3 times the quantity which will dissolve in an aqueous solution having the same salt concentration and pH as the plasma. Its greater solubility in plasma is

attributed to the action of the parathyroid hormone. Most conditions showing abnormalities in blood calcium which are not merely secondary to reduced plasma proteins or to primary changes in P are attributable to disturbances of parathyroid function. Injections of parathormone, or spontaneous hyper-secretion of the parathyroids met with in adenomata of these glands cause a marked rise in blood calcium, to 14-18 mg. %. This is associated with increased excretion in the urine and with extensive decalcification of the bones, which may lead to diffuse osteoporosis, multiple localized rarified cystic areas, and spontaneous fractures. There is often pathological deposition of calcium in other tissues and formation of renal calculi. There is muscular weakness and hypotonia. The P is usually low, but after large doses of parathormone it also may be high, indicating apparently a greatly increased solubility of Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>. Huge doses of vitamine D have a similar effect in animals. The condition may be cured by removal of the tumors. A similar high Ca-low P ratio may occur in myelomatosis and malignant metastases to the bones.

Hypoparathyroidism, which may occur spontaneously or after parathyroidectomy, causes a fall in blood calcium and a rise in P. If the Ca falls to 7 mg. %, tetany ensues. It is relieved by the injection of calcium salts or parathormone or by administration of acids. Alkalies have the opposite effect. Vitamine D is ineffective. (Tetany is not produced when the fall in Ca is due to protein depletion. Tetany may also be the result of alkalosis, without change in Ca; see section on acid-base balance.)

In *rickets* there is usually a low P, with an approximately normal Ca. Less often the P is about normal and the Ca is reduced. See section on Rickets.

In osteomalacia of adults, seen chiefly in pregnant women, the same conditions as in rickets appear to exist. In healing fractures P is often high, Ca about normal. High calcium has been reported in polycythaemia vera. Low Ca often occurs in chronic diarrhoea.

In advanced *nephritis with acidosis* there is a rise in P, even to 20 mg. %, with a reciprocal fall in Ca. It approximately parallels the rise in creatinine. Figures over 8 mg. % indicate impending uraemia. Tetany may occur, but is rare.

High P also occurs in cases of vomiting which lead to marked Cl depletion.

There is a transient fall in P of about 1 mg. % after the *ingestion of glucose*, while the latter is being removed from the blood stream. This is attributed to a combination of glucose with phosphoric acid, which appears to be an essential step in the storage and utilization of glucose. This fall in P does not occur or is lessened or delayed in diabetes,

and observations of blood P have been suggested to supplement those of the blood sugar in glucose tolerance determinations. A similar fall in P occurs after injections of insulin or epinephrin.

More information is obtained by a study of the total Ca and P metabolism than merely from blood analyses. This requires precisely regulated diets and quantitative estimations of Ca and P in both urine and feces over substantial periods and is impracticable except for research purposes.

Quantitative Determination of Phosphatase. (Method of King and Armstrong, 1934).—(1) In each of two test tubes (duplicate determinations) put 10 cc. of buffered substrate solution and place the tubes in the water bath at 37°C. for 5 minutes. Add to each 0.5 cc. of clear serum or plasma, mix, and incubate exactly 30 minutes. Then add to each 4.5 cc. of diluted phenol reagent and filter.

- (2) As controls prepare two other tubes in the same manner but add the diluted phenol reagent immediately after adding the serum.
- (3) Put 10 cc. of each filtrate in a series of test tubes and in a fifth tube put 10 cc. of standard phenol solution and reagent. (4) To each tube add 2.5 cc. of 20% sodium carbonate solution and place in the water bath for 5 minutes. (5) Read in the colorimeter, setting the unknown at 30 and moving the standard to match it. If the reading is over 30, set the unknown at 15. If over 00, repeat, diluting the serum with salt solution before hydrolysis.

The result is expressed as units per 100 cc. of serum, one unit being the activity required to liberate 1 mg. of phenol under the conditions of the test.

Calculation: Units of phosphatase per 100 cc. = reading of standard against test—reading of standard against control.

**Solutions.** (1) Buffered substrate.—Dissolve 1.09 Gm. of disodium phenylphosphate and 10.3 Gm. of sodium veronal in water to make 1 liter. Add a few drops of chloroform and store in the ice box.

- (2) Phenol reagent.—In a 1500 cc. flask dissolve 100 Gm. sodium tungstate and 25 Gm. sodium molybdate in 700 cc. of water and add 50 cc. of 85% phosphoric acid and 100 cc. of concentrated HCl. Boil for ten hours with a reflux condensor, the stopper of which is wrapped with tin foil. Add 150 Gm. lithium sulphate, 50 cc. of water and a few drops of bromine and boil for 15 minutes without a condensor. Cool and dilute to 1 liter. Before use dilute one part of this solution with two parts of water.
  - (3) A 20% solution of anhydrous sodium carbonate.
- (4) Standard phenol solution. Dissolve 1 Gm. of phenol (crystals) in N/10 HCl to make one liter. Put 25 cc. of this solution in a 250 cc. flask, add 50 cc. of N/10 NaOH and heat to 05°C. Add (hot) 25 cc. of N/10 iodine solution, stopper flask and let stand 30 minutes. Then titrate the excess of iodine with N/10 thiosulphate solution. Calculate from this figure how much the standard solution must be diluted to give a concentration of just 10 mg. in 100 cc. (The undiluted solution has about ten times this concentration.) One cc. of N/10 iodine corresponds to 1.567 mg. of phenol. Therefore, if the solution is exactly correct the 25 cc. of standard solution titrated will combine with 15.90 cc. of the N/10 iodine solution, and 9.04 cc. of N/10 thiosulphate will be required to neutralize the balance of the iodine. The volume to which 100 cc. of the strong standard must be diluted is 40 times the value of w in the

equation:  $w = \frac{25 \times (25 - No. cc. of thiosulphate used)}{15.06}$ . Store the diluted standard

### CHEMICAL EXAMINATIONS OF THE BLOOD

(5) Prepare daily the standard phenol solution and reagent as follows: Dilute 5 cc. of the diluted standard phenol solution and 15 cc. of (undiluted) phenol reagent with water to 50 cc.

Significance.—Phosphatase (phosphoesterase) is a ferment found in large amount in actively growing bone, which will split phosphate esters in vitro and which appears to bring about the deposition and absorption of calcium phosphate in bone. It is found in smaller amount in some other tissues and in the blood. The amount revealed by this method in normal blood ranges from 3 to 13 units. (The normal range as determined by Bodansky's method is from 1.5 to 4.5 units.) It is larger in children than in adults. It is increased (from 3 to 10 times the normal) in generalized osteoporosis, in hyperparathyroidism, and in the active stages of rickets and Paget's disease, even in cases in which the serum calcium and phosphorus are within normal limits. It may, therefore, be of considerable diagnostic value. An increase has also been reported in jaundice.

### Plasma Proteins

**Procedure.**—Oxalated plasma, which must be free from hemoglobin, is centrifugalized until clear.

Total protein.—I cc. of plasma is diluted to 50 cc. in a volumetric flask with 0.9% salt solution. Put I cc. of diluted plasma in a pyrex digestion tube, and proceed as under "Digestion." (Tube I.)

Total protein less fibrinogen.—To 1 cc. of plasma in a 50 cc. graduated cylinder add 48 cc. of 0.9% salt solution and 1 cc. of 2.5% calcium chloride solution. Mix and let stand for about an hour. Detach the fibrin clot with a fine pointed glass rod, transfer to a dry filter paper, squeezing out the fluid by rotating the rod. Put 1 cc. of filtrate in a digestion tube and proceed as below. (Tube 2.) (If only the total globulin content is desired, this step may be omitted.)

If only the fibrinogen content is desired, the clot (after the fluid has been completely expressed) is transferred to a digestion tube, and the procedure under "digestion" followed. The fibrin from r cc. of normal plasma contains from 0.4 to 0.6 mg. N.

Plasma albumin.—All procedures must be carried out at incubator temperature. To 1 cc. of plasma in a 50 cc. graduated cylinder add 30 cc. of 22.2% solution of anhydrous sodium sulphate, mix, and let stand in incubator 3 hours. Filter repeatedly through a fine grade of filter paper until perfectly clear. Transfer 1 cc. filtrate to a digestion tube. (Tube 3.)

Digestion.—(1) To each tube add 1 cc. of sulphuric acid digestion mixture and heat over a micro burner (either first adding a few chips of broken porcelain and a drop of caprylic alcohol, or shaking the tube gently throughout the heating) until the water is driven off and dense white fumes appear. (2) Cover mouth of the tube with a funnel, and heat more gently until the material is charred and the tube filled with dense white fumes. (3) Cool 30 seconds, and add drop by drop, Merck's superoxyl until the solution is water clear, (counting the drops). (4) Boil one minute. (5) Cool and dilute to 35 cc. (6) In a 50 cc. volumetric flask put 3 cc. of working nitrogen standard solution

containing 0.15 mg. N, r cc. of the digestion mixture, and about 25 cc. water. (7) To each tube add 15 cc. of Nessler's solution, diluting the standard to the mark with water. (8) Mix and read in the colorimeter.

Determine the non-protein nitrogen in the serum by the method previously given. For research accuracy the nitrogen in the superoxyl should be determined and subtracted, but for practical clinical purposes this is superfluous.

Calculation:

Per cent Protein = 
$$\left[ \left( \frac{\text{Stand}}{\text{Unkn.}} \times 0.15 \times \frac{100}{\text{V}} \right) - \text{NPN.} \right] \times \frac{6.25}{1000}$$

V = volume of plasma used: 0.02 in cases of total protein, and total protein less fibringen, and 0.0323 in case of albumin.

Total protein - albumin = total globulin.

Fibrinogen = total protein (tube 1) - protein in tube 2.

Albumin-globulin ratio = albumin/total globulin.

Determinations should be done in duplicate, particularly the globulin precipitation, which is the step at which error is most likely to occur.

Reagents. Sodium sulphate.—Dissolve 22.2 Gm. of the anhydrous salt in water in a 100 cc. volumetric flask at 37°C.

Acid digestion mixture.—To 10 cc. of 5% copper sulphate solution add 100 cc. sulphuric acid, and pour the mixture carefully into 100 cc. of distilled water, stirring constantly.

Significance.—There is from 0.25% to 0.4% fibrinogen in normal blood plasma. It is formed solely in the liver. Its normal function appears to be limited to the production of blood coagulation. It is increased in most acute infections and inflammations, and in conditions in which there is destruction of body tissue. It is reduced in cases of extreme liver injury, but may be normal or even increased in cases showing extensive but not maximal degrees of destruction of liver tissue. It is, therefore, of little value as a test of liver function.

Normal plasma contains 7% (6.3% to 8.0%) of total protein, of which 4.4% (3.8% to 5.2%) is albumin, and 2.6% (2.0% to 3.5%) is globulin (including 0.2 to 0.4% of fibrinogen.) The normal albumin-globulin ratio is 1.7 (1.45 to 2.2).

The plusma proteins do not appear to participate directly in the nutrition of the tissues. Their major known function is to control the distribution of fluid between the capillaries and the intercellular tissue spaces. They do not pass through the normal capillary endothelium (the intercellular fluids contain practically no protein), but exert a colloidal osmotic pressure or "oncotic" pressure (of 21 to 29 mm. Hg.) which tends to draw fluid from the tissues into the vessels. This, it is believed, normally counteracts the effect of the hydrostatic pressure within the vessels, which tends to force fluid into the tissues. In the arterial end of the

capillary loop the hydrostatic pressure prevails, and fluid passes into the tissues. In the venous end normally the colloidal osmotic pressure of the proteins is the greater, and the lost fluid is recovered. Albumin exerts about four times as great a colloidal osmotic pressure as globulin (per Gm.) and changes in it are of primary importance. Oedema commonly occurs in any condition in which there is a substantial fall in plasma albumin. The critical point is 2.5% albumin or 5.5% of total protein (Peters and Van Slyke).

Fluctuations in total protein occur as a result of changes in plasma volume. A marked increase occurs with dehydration, as after protracted vomiting or diarrhoea; in cholera, e.g., it may rise to 11%. A slight decrease (to about 6%) occurs in the first six months of pregnancy. A decrease occurs after protein starvation ("war oedema") and in a great variety of wasting diseases. Marked decreases in albumin occur commonly in "nephrosis," including amyloidosis, and in the active (nephrotic) stage of hemorrhagic nephritis, often falling to 2% and even to less than 1%. The globulin as a rule is not significantly reduced, and may even be increased. As a result the albumin/globulin ratio falls and may be reversed. One factor causing the low plasma protein is the loss of albumin in the urine, which in these conditions often amounts to 10 Gm. (up to 25 Gm. or more) per day. Malnutrition is also a factor.

Low plasma albumin and an inverted A/G ratio also occurs in advanced disease of the liver. There is much evidence that the plasma albumin (and possibly also the globulin) is synthesized in the liver.

The administration of sodium chloride markedly aggravates the oedema, and rigid salt restriction helps to control it. Nothing is known as to the mechanism of this action. Diets high in protein (90 to 120 Gm. per day), even in cases of active nephritis, raise plasma albumin substantially (but usually not to normal) and often result in elimination of the oedema and marked subjective improvement. These quantities do not appear to damage the kidney or lower renal function. The reserve protein of the body appears to be depleted in these conditions, and large amounts of protein may be taken and stored without increase in the N. excretion in the urine. However, albuminuria, a lowered plasma albumin, and a tendency to oedema persist, unless a spontaneous remission or recovery occurs.

Increase in globulin, especially fibrinogen, occurs in acute infections, including those associated with acute nephritis, and may partly mask the effect of the reduction in albumin. It is especially marked in kala azar (q.v.). Globulin is also increased in carcinomatosis and especially in

myelomatosis. This increase is a major factor in causing the accelerated sedimentation rate of the red cells seen in these conditions.

Oedema may occur, with normal plasma protein, as a result of increased intracapillary pressure (local stasis, the chronic passive congestion of myocardial failure); or of damage to the capillary walls which permits protein to leak into the intercellular spaces and to neutralize the effects of the colloidal osmotic pressure of that left in the vessels (inflammatory processes, angioneurotic edema). It may also follow the administration of excessive amounts of alkali.

#### Cholesterol

Procedure (Sackett).—(1) In a 25 cc. volumetric flask put 15 cc. of alcohol-ether mixture. (2) Add gradually with constant shaking 1 cc. of plasma or serum. (3) Hold in boiling water bath until it boils vigorously. (4) Cool in cold water, dilute to volume with alcohol-ether, and mix. (5) Filter through S. and S. fat free filter. (6) Pipette 5 cc. into a 50 cc. beaker, and (7) evaporate just to dryness over a boiling water bath. (Do not overheat.) (8) Extract residue twice with 2 cc. of boiling chloroform, decanting the chloroform into a 10 cc. glass stoppered graduate. (9) Cool and dilute to 5 cc. with chloroform. (10) In a similar graduate put 5 cc. of cholesterol standard solution containing 0.4 mg. (11) Add to each 2 cc. of acetic anhydride and (12) 0.1 cc. of concentrated sulphuric acid. (13) Mix and let stand in a dark place 15 minutes. (14) Read in colorimeter (in special cups sealed with plaster of Paris instead of cement). Calculation: If the standard is set at 15,

 $\frac{15 \times 0.4 \times 100}{\text{Reading} \times 0.2} = \frac{3000}{\text{Read.}} = \text{mg. cholesterol per 100 cc. (both free and bound)}.$ 

If whole blood is to be examined 50 cc. of alcohol-ether should be used for 1 (or 1.5 cc.) of blood. Moisture in the solutions causes turbidity. If the alcoholic extract is overheated, the final solution will show a brownish tinge which makes accurate readings impossible.

Reagents.—Alcohol-ether mixture. Mix 3 parts of anhydrous alcohol and one part of anhydrous ether (both redistilled).

Chloroform must be redistilled, anhydrous "reagent" chloroform.

Cholesterol standard: stock solution. Dissolve 0.4 Gm. in 250 cc. of anhydrous chloroform.  $\tau$  cc. =  $\tau$ .6 mg.

Working standard.—Dilute 5 cc. of the stock standard solution to 100 cc. with anhydrous chloroform. Keep in glass stoppered bottles. 1 cc. = 0.08 mg. 5 cc. = 0.4 mg.

Significance.—The normal figures for cholesterol in whole blood and serum usually range from 150 to 200 mg. per 100 cc. Very little is known as to the function of cholesterol in the body. It is supposed to be concerned in the absorption and metabolism of fat. Although an alcohol and not a fat, it is classed as a blood lipoid, (along with neutral fat, fatty acids, phosphatids, etc.) of which it normally constitutes about 20% to 40%. It

occurs partly as free cholesterol, (30% to 60%), and as cholesterol esters of fatty acids. As it is usually increased in conditions in which blood fat is increased, cholesterol determinations have commonly been employed as an indication of the amount of fat present. However, there is not a close parallelism between them, and in some conditions there is a divergence.

Blood cholesterol is little affected by the fat content of the *dict*, but it is increased by the administration of foods rich in cholesterol or in vegetable sterols. It is reduced in acute febrile *infections*, in most cases of *malnutrition* and cachexia, and in severe *anaemia* (although the blood fat may be high). In pernicious anaemia it may fall to 70 mg. %. It is slightly high in pregnancy. Increases have been reported in cases of cholecystitis and gall stones, (and in arteriosclerosis), but these findings have not been confirmed by later observations except when *obstructive jaundice* is present.

A marked increase in cholesterol, up to 350-600 mg. %, (and also of fat) is frequent in diabetes, particularly in severe cases with a tendency to ketosis, falling rapidly under insulin treatment. Less marked increases may be found in starvation acidosis, and in cases on a ketogenic (high fat) diet. Peters and Van Slyke suggest that high blood fat may in general indicate a response to a greater demand of the tissues for fat because of the lack or the unavailability of other fuel.

Marked increases of cholesterol (and all other lipoids) are seen in "nephrosis" and the nephrotic stage of active nephritis. In these diseases considerable cholesterol is excreted in the urine, and it constitutes the doubly refractile granules that may be seen in the renal tubular epithelial cells. In other types of nephritis blood cholesterol is usually not increased, and it may fall sharply as uraemia is approached, even though blood fat is high. High cholesterol has also been reported in cases of generalized xanthomatosis, both in the diabetic and non-diabetic type, and in some cases of Christian-Schüller disease. It is much increased in myxoedema (an important diagnostic point), and is diminished in hyperthyroidism.

# Quantitative Determination of Alcohol in Body Fluids

### BOGEN METHOD

Method in brief.—Draw air through 1 cc. of specimen to be examined and then through 5 cc. of Anstie's reagent (dichromate-sulphuric acid) while both are immersed in a boiling water bath. Measure alcohol

present by comparing color of reagent with previously prepared standards containing known amounts of alcohol. Color varies from reddish yellow to greenish blue.

In our experience the majority of men showing over 2 mg. alcohol per cc. of blood or urine had been pronounced intoxicated by the clinical examiner, acting independent of the laboratory. The vast majority below the two milligram level had in like manner been pronounced not intoxicated. Thus the 2 mg. concentration was considered the dividing line, as a rough approximation. No person with less than 1 mg. of alcohol in 1 cc. of urine gave clinical evidence sufficient to justify a diagnosis of acute alcoholic intoxication. Above this concentration, the frequency of the clinical symptoms of drunkeness increased rapidly with the amount of alcohol found. 75% of men who showed blood alcohol concentrations of 1.5 mg. to 2.0 mg. per cubic centimeter were, by independent clinical examination, judged to be intoxicated. Of the men showing 2.5 to 3.0 mg. concentrations 85%, while all of those showing 3.5 to 4.0 mg. were judged intoxicated and 30% were in coma. On experimental animals 6.0 mg. kills.

The susceptibility of the brain to alcohol varies little between one normal individual and another. The apparent variation in susceptibility in different individuals is thought to be governed more by the rate of absorption on one hand, and oxidation, exhalation, and excretion on the other, than to any inherent resistance of the cell protoplasm to the toxic effects of alcohol. Haggard and Greenberg ( $\tau_{037}$ ), however, found that the lethal concentration in the blood for rats was higher (12 mg. per cc.) if the blood sugar was 200 mg. per cent than if it was low (8.0 mg. per cc. with a blood sugar of 70 mg. per cent).

Method.—Blood is collected by venepuncture and oxalated as for other chemical procedures (2 mg. oxalate per cc. of blood). When cleansing the arm with alcohol or tincture of iodine prior to puncture, allow to dry completely to avoid fouling the needle.

Three tubes are set up in a row and connected for aeration. In the tube farthest from the suction pump, place 1 cc. of blood, urine, or other fluid to be examined, 1 cc. Scott-Wilson reagent (for fixation of acetone), 3 to 5 cc. of distilled water, and 5 to 10 drops of liquid petrolatum (optional) to prevent foaming. The center tube is empty and is placed in series to guard against foaming over directly into the reagent tube. The tube nearest the suction pump contains 5 cc. of Anstie's reagent. Place connected tubes in a boiling water bath and aerate gently at moderate speed for 10 minutes. Compare reagent tube with known standards.

The method depends upon the fact that, when a solution of potassium bichromate in sulphuric acid is treated with alcohol, the alcohol is oxidized, largely to acetaldehyde,

while the bichromate is reduced with the formation of chromic sulphate according to the following equation:

$$K_2Cr_2O_7 + {}_4H_2SO_4 + {}_3C_2H_6OH = {}_3C_2H_4O + K_2SO_4 + Cr_2(SO_4)_3 + {}_7H_2O.$$

The green color in the completed reaction is due to the production of chromic sulphate. Scott-Wilson reagent.—Dissolve 1 Gm. of mercuric cyanide in 60 cc. of distilled water, using a heavy walled glass jar. Add a solution of 18 Gm. of sodium hydroxide in 60 cc. of water, stir vigorously with a heavy glass rod, add 0.290 Gm. of silver nitrate dissolved in 40 cc. of water. If turbid, allow to stand 2 or 3 days, decant the clear supernatant fluid. This keeps several months.

Anstie's reagent.—Dissolve with the aid of heat in water bath 0.666 Gm. of potassium bichromate (CP) in 100 cc. concentrated sulphuric acid (CP). This will be labeled "Concentrated Anstie's" and used in preparing the standards. Dilute with equal parts distilled water for use in the test.

Preparation of standards.—Make a 1% solution of alcohol by pipetting 1.200 cc. of absolute alcohol at 15°C. into distilled water. Make up volume to 100 cc. in volumetric flask. Prepare a series of 11 tubes (12 by 150 mm.), labeled 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mg. per cc., respectively. Pipette the 1% alcohol into each tube to represent the above figures. Make up volume in each tube to 2.5 cc. with distilled water. Add to each tube 2.5 cc. of concentrated Anstic's reagent (2% per cent potassium dichromate in sulfuric acid). Seal tubes by heating and drawing from top.

Example.—Tube marked "o.5 milligram per cubic centimeter" contains:

	Cubic
ı per cent alcohol	0.05
Distilled water	1.07
Concentrated Anstie's reagent	2.50
Total	5.00

Cultin

These standards are reliable for approximately 3 months, after which time the color in the higher concentrations of alcohol breaks down and begins to fade.

It has been our routine in cases which seem to have 3 mg. alcohol per cc. of blood or higher to repeat the test using only 0.5 cc. of blood and to multiply by 2 for the final reading. This has been considered advisable as matching of color, casy in the lower, is more difficult in the higher standards. One is also approaching the limit of capacity of the reagent at the 4 mg. concentration.

A small color change approximating the 0.5 mg. standard is, in our experience, produced by all normal bloods in the above method. This change is produced by some volatile organic constituent of the blood, probably, at least partially alcohol, for there is known to be alcohol present in minute quantities in the tissues normally. Therefore a change corresponding to the 0.5 mg. standard should not be taken to mean that the individual being examined has imbibed alcohol.

Non-volatile material, glycerin, phenol, cresol, etc., added directly to the reagent, produce the same color change as alcohol. Ether as well as other volatile organic substances not encountered in the body fluids also react similarly. Therefore, care and

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cleanliness with instruments and glassware as well as in the performance of the test are essential to the integrity and reliability of the procedure.

Because of the reaction, with the reagent, of substances in cork and rubber stoppers, tubed standards, which are to be kept for some time and which may be inverted, should be sealed as directed above by heating and drawing out the glass.

#### **Bromides**

The method of Wuth (1927) as modified by Katzenelbogen and Czarski (1934) affords a simple and quick estimation of the quantity of bromides in the blood, which is useful in detecting intoxication from overdosage with the drug and in controlling treatment.

(1) Add 1 cc. of clear serum to 2.6 cc. of 5% trichloracetic acid in a small test tube, mix and filter through a small filter. (It may be filtered immediately; no bromide is lost in the precipitate.) (2) Transfer 1 cc. of filtrate to a comparator tube about 10 mm. in diameter and add 0.2 cc. of 0.5% gold chloride solution. (3) Compare at once with the series of standard tubes, previously prepared.

Standard Inbex.—Prepare a stock solution containing 0.5% sodium bromide in 0.6% salt solution. Into each of a series of 12 test tubes put quantities of stock bromide solution, beginning with 0.5 cc. in the first tube and increasing the quantity by 0.5 cc. in each succeeding tube, to 6.0 cc. in the twelfth tube. Make two additional tubes containing 7 cc. and 8 cc. respectively. Add enough 0.6% salt solution to each tube to bring the volume to 10 cc. and mix. These correspond to serum bromide concentrations ranging from 25 mg. per 100 cc. in the first tube to 300 mg. in the twelfth and 400 mg. in the fourteenth tube.

Put 1 cc. of each of these dilutions in a series of comparator tubes identical in diameter with that used above, and add to each 2.6 cc. of 5% trichloracetic acid and 0.72 cc. of 0.5% gold chloride solution; mix.

These standard tubes are semi-permanent, in that they will keep their color for from one week to six months if they are covered with paraffin oil, tightly stoppered and kept in the dark. If it is desired only to determine the existence and approximate degree of bromide intoxication, it suffices to prepare four dilutions, containing 2, 3, 4 and 6 cc. of stock solution, each diluted to 10 cc., corresponding to 100, 150, 200 and 300 mg. per 100 cc. of blood.

Normal serum gives no color. A reading over 150 mg. per 100 cc. indicates over-dosage and is often associated with symptoms of intoxication.

Bromides may be detected in the urine by shaking up with animal charcoal, filtering, and adding to 5 cc. of filtrate 1 cc. of 20% trichloracetic acid solution and 1 cc. of 0.5% gold chloride solution. If no brown color appears, a test of the blood is useless.

# DISTURBANCES OF ACID-BASE EQUILIBRIUM

The blood plasma and other body fluids have a slightly alkaline reaction. The normal metabolic processes result in the continuous production of acid end-products which tend to make the reaction of the blood more acid. The physiological activities of the body, however, are so adjusted that they keep the variations in the reaction of the plasma within narrow limits. This is accomplished first, by the immediate virtual neutralization

of these acid substances as they are formed in the tissues, and second, by the elimination of the excess of acid radicals by the lungs and kidneys. Following the ingestion of unusual amounts of acid or alkali or as a result of disease this neutralizing mechanism may be so overtaxed, or its activities so disturbed, as to result in a significant shift in the reaction of the blood. An acidosis, or less often an alkalosis is the result.\* Any marked shift in the reaction is fatal if not quickly corrected or compensated. Such shifts are associated with characteristic clinical symptoms and with changes in the blood, urine, and expired air which make possible their early recognition. To recognize these conditions and treat then intelligently it is essential to be familiar with the mechanisms which normally maintain this reaction at so nearly constant a level, and the usual ways in which it may be disturbed.

The strength (intensity) of an acid in solution, as distinguished from its quantity, or titratable acidity, depends upon the degree to which it is dissociated into hydrogen ions (H+) and acid ions (A-). That is, it depends upon the hydrogen ion concentration of the solution. This is expressed in terms of normal acidity, as a fraction of which the numerator represents the quantity of dissociated H ions present, and the denominator the quantity of H in a normal solution (I Gm. per liter). In the case of a strong acid like HCl, which is almost completely dissociated in dilute solution, the reaction (H+ concentration) of a one hundredth normal solution would be nearly o.o. N. In case of a weak acid like H<sub>2</sub>CO<sub>3</sub> the H<sup>+</sup> concentration of a 0.01 N solution would not be 0.01 N, but very much less than this (circa 0.00007 N) because only a small fraction of its II is ionized. In pure water, which is dissociated to a slight extent into H+ and OH- ions, and which is neutral because the number of H+ and OH- ions are equal, it has been determined that the H<sup>+</sup> concentration is 0.000000  $\iota$  N, or  $\iota \times \iota o^{-7}$ . To avoid the use of clumsy fractions it is customary to express the reaction of such solutions in terms of the negative logarithm of the fraction, which is indicated by the symbol pll. Thus the pH of water is  $-\log (0.0000001) = -\log (1 \times 10^{-7}) = -(-7) = 7$ , the neutral point. More acid solutions have a higher H+ concentration, and a smaller negative log (pH). The pH of a o.o. N solution of HCl, having a H ion concentration of (nearly) o.o1 N, would be  $-\log(0.01)$ ,  $= -\log(1 \times 10^{-2})$ , = -(-2), or 2. Alkaline solutions have a higher pH than 7. Thus the pH of a 0.01 N solution of NaOH would be approximately 13.

The pH of the blood and tissues usually ranges from 7.3 to 7.5, and the maximum range compatible with life is from 7.0 to about 7.8.

The possibility of maintaining the reaction of the blood within such narrow limits depends upon the presence of buffers in the blood. A buffer is a substance which lessens the change in reaction which follows the

\*The term "acidosis" is a misnomer, since during life the body fluids never acquire an acid reaction. There is at most only a shift toward the neutral point. We retain the term, however, because it is in general use.

addition of acid or alkali to a solution, as compared with the change which would occur if no buffer were present. All buffers are mixtures of a weak acid and its basic salts, or a weak base and its acid salts. The buffers in the blood which are of chief importance are  $\rm H_2CO_3$  and its bicarbonate salts, and haemoglobin (a weak acid at pH 7.4) and its basic salts. The plasma proteins have some buffer action. The monobasic and dibasic phosphates also constitute a buffer, but their quantity is too small to exert an appreciable effect in the blood.

The mechanism of the action of a buffer may be best explained by an example (from Peters and Van Slyke). If enough HCl is added to a neutral unbuffered solution to give a concentration of 0.05 N, the H<sup>+</sup> concentration will be nearly 0.05 N, and the pH will be lowered from 7.0 to about 1.3, a drop of 5.7 units. If instead it is added to a solution containing both H<sub>2</sub>CO<sub>3</sub> and NaHCO<sub>3</sub> in 0.1 M concentration, the following reaction will take place: HCl + NaHCO<sub>3</sub> = NaCl + H<sub>2</sub>CO<sub>3</sub>. The strong acid will be replaced by a corresponding amount of the weak acid, H<sub>2</sub>CO<sub>3</sub>, while half the NaHCO<sub>3</sub> is used up. The ratio NaHCO<sub>3</sub>/H<sub>2</sub>CO<sub>3</sub> will fall from  $\frac{1}{2}$  to 0.5/1.5, and the pH will fall (the "neutralization" of an acid by a buffer is not complete), but only from 6.1 to 5.3, or 0.8 units.

In the blood,  $CO_2$  exists in two forms; partly combined as bicarbonates of the blood bases (chiefly sodium), and partly free as  $CO_2$  or  $H_2CO_3$  in physical solution in the plasma. Normally the ratio of combined  $CO_2$  to free  $CO_2$  is about 20 to 1.

The reaction of the blood (pH) depends directly on the ratio between the bicarbonate content of the plasma and its  $CO_2$  tension (the amount of  $CO_2$  in physical solution).

This can be calculated, if the values of these two factors are known, from the Henderson-Hasselbalch equation, which in its simplified form is given by Peters and Van Slyke as follows:

$$pH = 6.10 + \log \frac{112CO_3}{H_2CO_3} = 6.10 + \log \left(\frac{mM(CO_2)}{0.0301p}\right)$$

In this formula  $mM(CO_2)$  represents the total  $CO_2$  (free and combined) in the plasma, expressed as mM; and p the  $CO_2$  tension of the plasma in mm. of Hg. Numerical values determined by calculation from experimental data have been substituted for pK' and  $\alpha$  in the original equation. (These values are valid only for serum or plasma at  $38^{\circ}C$ .)

It is obvious that if any two of these three factors, pH, CO<sub>2</sub> content, and CO<sub>2</sub> tension, can be directly measured, the third can be calculated from the equation, or more simply from nomographic line charts which have been constructed according to the terms of the equation. Of these the CO<sub>2</sub> content is the most readily determined, by gas analysis (page 694). The pH can be determined directly by the colorimetric method of Cullen (1922), or one of its modifications. Determination of the CO<sub>2</sub> tension is more difficult,

but it can also be determined directly by the method of Van Slyke et al. (1032) by measuring the CO<sub>2</sub> tension in a small bubble of air which has been brought into equilibrium with 9 volumes of whole blood. It can be calculated by Eisenmann's method (1926) from determinations of both the CO<sub>2</sub> content of the plasma (or serum), and the CO<sub>2</sub> combining power of plasma when saturated with CO<sub>2</sub> at two or more different tensions. It may also be determined indirectly by measuring the CO<sub>2</sub> tension of the expired air (by the Haldane or Fridericia methods, without rebreathing), which is usually identical with the tension in arterial blood. Details of these procedures will not be given as they require equipment available only in large laboratorics in which special text books on the subject will be available.

The amount of combined CO<sub>2</sub> (bicarbonate) present depends directly on the amount of base in the blood which is not combined with stronger acids. If base is liberated when such an acid is eliminated by combustion or excretion, it immediately combines with free CO<sub>2</sub> in the plasma. As a result the ratio BHCO<sub>3</sub>/H<sub>2</sub>CO<sub>3</sub> rises and the pH is slightly increased. If free acid is produced or absorbed, it immediately robs some of the bicarbonate of its base, which neutralizes the acid, and increases the free CO<sub>2</sub> in the plasma. The bicarbonate/CO<sub>2</sub> ratio is lowered, and the pH falls. This effect is largely counteracted by prompt elimination of the excess of CO<sub>2</sub> by the lungs, but some reduction in the plasma bicarbonate remains. Because the plasma bicarbonate is the most immediately available and quantitatively the most abundant store of base which can be utilized for neutralization of acids, it is commonly spoken of as the alkali reserve of the blood. Simple methods are available for its estimation. This alone does not suffice to determine the pH, but in the commoner types of disturbance of acid-base equilibrium, as in diabetes and nephritis, changes in pH usually follow, roughly, changes in alkali reserve. While there are exceptions which will be discussed later, determination of the alkali reserve remains the most valuable single practicable procedure for the estimation of these disturbances.

The quantity of  $CO_2$  normally present as bicarbonate in the plasma of oxygenated blood (pH of 7.4,  $CO_2$  tension of 40 mm.) is about 24 to 25 mM. or 56 volumes per cent. (The total  $CO_2$  is about 1.2 mM., or 2.7 vol. % higher. 1 mM.  $CO_2$  = 22.26 cc., or 2.226 vol. %.) The maximum normal range for total  $CO_2$  is usually given as from 55 to 75 vol. %. The figures for whole blood are about 20% lower, as there is less bicarbonate in the cells than in the plasma. The terms acidosis and alkalosis are commonly used to include conditions in which there is a significant alteration in the alkali reserve even though there is little or no change in the pH.

The amount of  $free\ CO_2$  present in the plasma depends directly upon the concentration or tension of  $CO_2$  in the atmosphere with which it is in

equilibrium. This depends upon the balance between its rate of production in the tissues and its rate of excretion in the expired air. Since the rate of production of  $CO_2$  is not susceptible to direct control, maintenance of a normal  $CO_2$  tension (and a normal pH) must depend primarily on the ventilation rate of the lungs. This is accelerated when  $CO_2$  tension is increased, as by breathing air to which  $CO_2$  has been added, and is usually diminished when  $CO_2$  tension is lowered. However, it appears to be not the increased  $CO_2$  tension itself, but the lowered pH which usually accompanies it, which causes the increase in ventilation rate, presumably by stimulating the respiratory center.

The haemoglobin of the red cells, with its basic salts, is a buffer second in importance only to the CO2 and bicarbonate of the blood. It is of chief importance in minimizing the changes in reaction which occur with changes in the CO2 content of the blood. This action is in part that of a simple buffer. When the CO2 content (and tension) of the blood is increased in its circulation through the tissues, it becomes in effect a stronger acid, and robs the haemoglobin of a small part of the base combined with it, to form bicarbonate. This tends to make the bicarbonate CO2 ratio and the pH higher than it would have been had no haemoglobin been available. However, the changes in the intensity of the acidity of haemoglobin which occur with oxygenation and reduction far outweigh in their neutralizing effect its action as a simple buffer. As arterial blood circulates through the tissues it simultaneously gives up O to the tissues and receives CO<sub>2</sub> from them (in about the proportion of 10 to 8). When haemoglobin is reduced by loss of O, it becomes less strongly acid, and as a result liberates base, which is sufficient to neutralize (by forming bicarbonate) about  $\frac{5}{8}$  of the CO<sub>2</sub> received, without any change in pH. The acidifying effect of the remaining 38 of the CO2 is largely offset by the buffer action of the haemoglobin and the other buffers, so that with an average increase of 5 vol. % in the CO2 content of the venous blood, the pH falls only about 0.02 points.

The bicarbonate thus newly formed within the red cells raises the ratio of bicarbonate/Cl in the cells to a point above that in the plasma. As a result there is a diffusion of  $-HCO_3$  ions from the cells into the plasma and of -Cl ions from the plasma into the cells until the ratios are equalized (Donan's equilibrium). The basic ions (in the red cells chiefly potassium) can not pass through the cell membrane. This passage of  $-HCO_3$  ions into the plasma equalizes the bicarbonate/ $CO_2$  ratio, and therefore the pH, in the cells and plasma, and thus makes the buffer effect of the haemoglobin available to the whole blood, and not merely to the intracellular fluid, as would be the case if this diffusion could not occur. Incidentally, with the liberation of base by the haemoglobin and the simultaneous diffusion of Cl into the cells, the osmotic pressure within the cells

rises, and water is drawn from the plasma into the cells, causing them to swell, until osmotic equilibrium is restored.

In the lungs the reverse process takes place. The haemoglobin after oxygenation becomes more strongly acid, and abstracts base from much of the bicarbonate present, liberating a corresponding amount of CO<sub>2</sub>. This immediately diffuses out into the air in the alveoli until equilibrium is established, usually at a tension of about 40 mm. of Hg (2.7 vol. %, 1.2 mM. per liter). There is a simultaneous passage of Cl and water from the cells to the plasma and some shrinkage of the cells. The same phenomena occur when venous blood is exposed to the air in vitro, except that the blood loses all its CO<sub>2</sub>. It is obvious that in severe anaemia the buffer action of the blood is reduced, and acidosis more readily established.

In addition to CO<sub>2</sub>, other acids are produced in the course of normal metabolism. Some phosphoric and sulphuric acid are formed by the catabolism of protein. After violent exertion lactic acid is produced in the muscles, and if the diet is high in fat and low in carbohydrate, substantial amounts of ketone acids are produced. These are immediately neutralized by base from the bicarbonate and other buffers, as above described, and notable lowering of the pH is prevented by elimination of the liberated CO<sub>2</sub> by the lungs.

The total amount of acid which can be buffered by the blood in this way before an intolerable degree of acidosis is reached is substantial. Peters and Van Slyke estimate that to reduce the pH of 1 liter of blood from 7.4 to 7.0, about 28 milliequivalents of acid will be consumed. Of this about 18 mE. are buffered by the bicarbonate alkali, 8 mE. by the haemoglobin alkali, and 2 mE. by the alkali of other buffers, chiefly the plasma proteins. As the total blood volume is somewhat over 5 liters, it would be able to buffer about 150 cc. of N acid. The total buffer power of the body tissues has been estimated to be about 5 times that of the total blood, or about 750 cc. additional N acid. Both together are equivalent to about 15 cc. of N acid per kilo of body weight. Much of this neutralizing power depends upon the elimination of the CO<sub>2</sub> liberated by the bicarbonate, and is not merely the result of simple buffer action.

This neutralization is accomplished only at the cost of lowering the alkali reserve. To prevent dangerous depletion of the alkali reserve it is essential that the acid radicals be eliminated without losing a corresponding amount of base. With rest and adequate O supply the lactic acid is speedily oxidized to CO<sub>2</sub> and water, or is resynthesized into glycogen, and the ketone acids may be largely or entirely oxidized, the base so liberated remaining in the plasma as bicarbonate. The maintenance of a normal alkali reserve, however, depends mainly upon the selective excretory capacity of the kidneys. The reaction of the urine is normally much more acid than that of the blood. This is due to the ability of the kidney to excrete weak acids (hydroxibutyric) and acid salts, and hold back a large part of the base with which they are combined in the plasma. Normally

the acid phosphates are the most important vehicle for this acid elimination. In the blood about 80% of the phosphate is in the form of dibasic phosphate. In strongly acid urine nearly all is excreted as monobasic phosphate, so that nearly half the base required to neutralize the phosphoric acid while it was in the blood is retained as bicarbonate and replenishes the alkali reserve. If considerable amounts of ketone acids are present in the blood (always combined with base), as in diabetic acidosis, they are excreted in the urine and as much as 50% of that excreted may be as free acid. The total amount of unneutralized acid (titratable acid) so excreted daily varies greatly, but normally is equivalent to 20-40 cc. of N acid. In severe acidosis it may equal 150 cc. of N acid.

If in spite of this acid excretion the blood bases begin to be depleted, the kidney begins to exercise its second important function in preventing loss of base. It breaks up urea with the production of ammonia, and utilizes this ammonia instead of fixed base for the neutralization of much of the acid radicals which it excretes. Even strong acids like HCl and  $\rm H_2SO_4$  may be excreted as ammonium salts. The normal daily excretion is about 30–50 cc. of N NH<sub>3</sub>, and in severe acidosis it may rise to 500 cc. Ammonia can not be utilized for the neutralization of acids in the body fluids. Only minute traces can be demonstrated in the blood.

The sum of the titratable acid + ammonia measures the excess of fixed acid over fixed base excreted, and serves as a rough measure of the bicarbonate content of the plasma in diabetic acidosis.

If, on the other hand, there is an excess of fixed bases in the blood, which rarely occurs except after ingestion of alkalis, the titratable acid and ammonia diminish or disappear, and bicarbonate may be excreted in the urine. The urine may then become a little more alkaline than the blood.

Disturbances of acid-base equilibrium may depend primarily upon changes in the quantity (tension) of CO<sub>2</sub> in the blood (disturbances of the ventilation rate), or upon disturbances of the relation of the blood bases to acids other than carbonic. The causes of the disturbances in these two groups of conditions are entirely different, as is the treatment. An understanding of these conditions is facilitated by a study of Fig. 172, which is taken from Van Slyke.

Normally the pH of the blood is automatically kept at a nearly constant level by changes in the ventilation rate of the lungs, which are brought about by the action on the respiratory center of slight changes in the pH of the blood. Thus a fall in pH stimulates the center, increases the ventilation rate, lowers the CO<sub>2</sub> tension, and raises the pH. If other

factors interfere with this regulatory process, a shift in the reaction will occur. Thus a diminished ventilation rate may occur if the sensitiveness of the respiratory center is reduced by morphine, or if breathing is disturbed mechanically, as in emphysema or obstruction of the respiratory

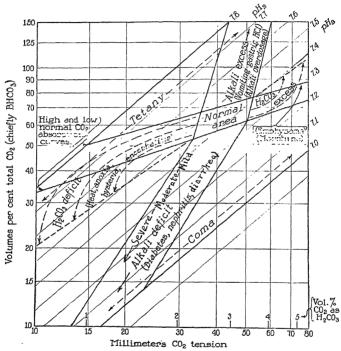


FIG. 172.—Chart showing changes in CO<sub>2</sub> content, H<sub>2</sub>CO<sub>3</sub> content, CO<sub>2</sub> tension and pH of the blood plasma in conditions of carbonic acid excess and deficit and of alkali excess and deficit. (Courtesy of D. D. Van Slyke and the New York Academy of Medicine Bulletin.)

passages. Then the  $CO_2$  tension rises and the pH falls. There is a primary  $CO_2$  excess. This is partly compensated by an increased excretion of acid radicals (PO<sub>4</sub>, Cl, etc.) and ammonia in the urine. The base so liberated neutralizes some of the excess of  $CO_2$ , raises the BHCO<sub>3</sub>/ $CO_2$  ratio, and partly corrects the fall in pH. This results in an increase in the plasma bicarbonate, in spite of the presence of some acidosis. Treat-

ment aimed directly and solely to correct the acidosis in these conditions is not necessary, as death from lack of O will occur before the acidosis becomes dangerous in itself. Administration of alkali is not indicated. Dyspnoea on exertion is the chief symptom.

Hyperventilation of the lungs reduces the CO<sub>2</sub> tension and raises the pH, even to the point of causing tetany (7.6+). There is a primary CO<sub>2</sub> deficit. This may occur as a result of breathing air with a low O tension (high altitudes), of hot baths, fever, hysteria, certain organic nervous diseases, and even by voluntary effort. A similar disturbance (high pH, low bicarbonate) has been reported after deep radiation therapy. It is partly compensated by arrest of renal excretion of acid and ammonia and by the increased excretion of fixed base. This reduces the alkali reserve, despite the presence of alkalosis. The logical treatment is to reduce the sensitiveness of the respiratory center by morphin, and to give salt and large amounts of fluid. In cases of tetany due to alkalosis there is no significant change in the blood calcium.

A primary alkali excess may occur as a result of overdosage with alkali, or loss of HCl from protracted vomiting (or gastric lavage) as in pyloric obstruction. The ratio BHCO<sub>3</sub>/H<sub>2</sub>CO<sub>3</sub> rises, and with it the pH. A plasma CO<sub>2</sub> content of 122 vol. % has been reported in pyloric obstruction. If the disturbance is marked, tetany may occur. The condition is compensated in part by slowing the respiration and raising the CO<sub>2</sub> tension, and in part by a lessened excretion of acid and ammonia, and by the excretion of sodium bicarbonate in the urine. In cases due to vomiting, dehydration and loss of electrolytes may be more serious than the alkalosis, and but little base is excreted. Treatment consists in overcoming the dehydration and reestablishing the urine excretion by injections of hypertonic salt solution. The kidney is then able to excrete the excess of alkali, and administration of acid is unnecessary.

The differentiation of an alkalosis due to primary alkali excess from an acidosis due to primary  $CO_2$  excess can not be made simply from an estimation of the alkali reserve, since this is increased in both conditions. If it depended solely on laboratory procedures it would be necessary also to determine either the pH or the  $CO_2$  tension. Practically this is rarely necessary, as the conditions can usually be differentiated on clinical grounds alone.

A primary alkali deficit is the most common and most important type of disturbance of acid-base equilibrium. It may be due directly to loss of alkali in the stools after severe diarrhoea, as in cholera and in infantile diarrhoeas. It can be produced by the administration of free acid or

acidifying salts. The similarity of the symptoms of dogs poisoned with acid (particularly the dyspnoea) to those of patients in diabetic coma led to the recognition of acidosis as the cause of the latter. It occurs temporarily after severe exercise. It may occur in diabetes, and in the acute ketoses of children as a result of retention of ketone acids. It occurs in starvation, and in cases on a ketogenic diet. In conditions associated with vomiting but without much loss of free HCl, a ketosis with alkali deficit commonly develops. In children this may be far more intense than in case of simple starvation. A fall in pH and in plasma bicarbonate occurs during general anaesthesia. It may occur (most markedly in venous blood) in circulatory failure, although conditions are complicated by varying degrees of CO<sub>2</sub> excess. It may occur in advanced renal insufficiency as a result of loss of selective secretory power, so that less acid is excreted and more fixed base escapes.

In all these conditions the distinctive feature is the lowered plasma bicarbonate and lowered pH. The mechanism for compensating this disturbance has already been discussed; hyperpnoea with a lowering of the CO2 tension; excretion of acid salts and even free hydroxibutyric acid in the urine; and the substitution of NH3 for fixed base in the salts excreted. These conditions are usually associated with a profuse diuresis which may lead to a dehydration and salt depletion which is almost as serious as the acidosis. Treatment of diabetic acidosis consists first in the administration of insulin, usually with glucose, to secure combustion of the ketone acids; and, second, the administration of salt solution to restore salts and fluid and maintain urine secretion. The base liberated by the oxidation of the ketone acids largely replenishes the alkali reserve, and if renal function is good, any remaining excess of acid can be excreted in the urine. A normal reaction can usually be restored in this way without administration of alkali. Recovery can be hastened, however, if sodium bicarbonate is also administered in moderate amounts. This seems advisable as a rule, particularly if it can be guided by repeated determinations of the plasma bicarbonate. Excessive amounts must be avoided, as they may cause a damaging degree of alkalosis. The same principles apply to treatment in the other conditions.

In the acidosis of renal insufficiency conditions are complicated by the inability of the kidney to excrete selectively excess of acid or alkali, and to readjust the relative quantities of the electrolytes. The ability to utilize ammonia to neutralize acid radicals is also lost. There is a retention of fixed acids (PO<sub>4</sub>, SO<sub>4</sub>, possibly others), and a depletion of fixed base. Here special care is required to adjust properly the dose of NaHCO<sub>3</sub>

and NaCl administered so as to restore normal proportions as nearly as possible. As the condition is a terminal one any benefit secured can be only temporary and symptomatic. (See also Table, p. 692).

Differentiation of a primary alkali deficit from a primary CO<sub>2</sub> deficit, both of which show a reduction in the alkali reserve, can usually be made on clinical grounds alone. If this can not be done, and if active treatment is needed, determination of the pH or CO<sub>2</sub> tension is important, as the treatment indicated in the two conditions is entirely different.

### Clinical Methods

Whenever a disturbance of the acid-base equilibrium is suspected a determination of the CO<sub>2</sub> combining power, or the CO<sub>2</sub> content of the plasma, should be carried out if facilities are available. If they are not, helpful information may still be obtained from the simpler procedures which are next described. The table on page 692, taken from Van Slyke, shows the relative significance of these indirect tests, and gives the values which may be expected normally, and in acidosis of varying severity.

Marked *ketonuria* in cases of starvation, inadequate carbohydrate intake, or diabetes mellitus, indicates the presence of an acidosis, but does not give an accurate estimate of its severity.

If renal function is normal, a high ammonia N/urea N ratio, and particularly an increase in the sum of titratable acid + ammonia in the urine is indicative of acidosis, and a rough measure of its severity in disturbances of the primary alkali deficit type. The  $CO_2$  combining capacity of the plasma can be estimated roughly from it by the formula of Fitz and Van Slyke:

$$CO_2$$
 capacity =  $80 - 5\sqrt{\frac{D}{W}}$ 

D = titratable acid + ammonia (in cc. of N/10 acid) excreted in 24 hours.

W = body weight in kilos.

The tolerance for bicarbonate (Sellards) is a simple procedure of considerable practical value as a test for acidosis when more precise methods are not available. Give by mouth (or intravenously if necessary) 5 Gm. of sodium bicarbonate in 100 cc. of water. After ½ hour secure a specimen of urine and test the reaction with litmus. If still acid, give an additional 5 Gm. of bicarbonate, and repeat until the urine is alkaline, boiling the specimen if the reaction is doubtful. In normal individuals 10 Gm. usually produces an alkaline urine, and 0.5 Gm. per kilo always does so. In acidosis larger amounts are required. A negative result (indicating absence of acidosis) is usually reliable. A positive result is not dependable in renal insufficiency, or other conditions in which the electrolytes are depleted. In these conditions caution is necessary, as an alkalosis may be produced by overdosage before the urine becomes alkaline.

The alveolar CO<sub>2</sub> tension, as determined by Marriott's method, approximates that in venous blood, which is normally about 45 mm. of Hg, about 5 mm. higher than in arterial blood. It is of value in that from it the approximate bicarbonate content of the plasma usually can be inferred. In a thin walled rubber bag of 1500 cc. capacity (like the inner bag of a foot ball) put one liter of air (600 cc. for infants), and clamp the exit

Comparison of Indirect Tests for Primary Alkali Depicit with Direct Dependations of Plasma CO<sub>2</sub> or Bicarbonate Van Slyke

			CORRESI	ONDING RESULTS OF	CORRESPONDING RESULTS OF INDIRECT TESTS FOR ACIDOSIS	ACIDOSIS	
CONDITION OF	(a) PLASMA CO <sub>2</sub> IN VOL., % (b) PLASMA	24 hour excr açid +	24 hour excretion! of 0.1 N acid + ammonia	Carbon dioxide of alveolar air		Sodium bicarbona urine	Sodium bicarbonate required to turn urine alkaline
SUBJECT	BICARBONATE IN MM.	(a) cc. per kilo (b) cc. per 60 kilo subject	Reliability in diabetes	(a) Tension in mm. (b) Approximate %	Reliability in diabetes	(a) Gm. per kilo <sup>3</sup> (b) Approximate Gm. per 60 kilo subject	Reliability in diabetes
Normal resting adult; extreme limits.	resting (a) 73-55 extreme (b) 31-23	(a) 0–27 (b) 0–1600	Good	(a) 53–35 mm. (b) 6.8–4.7 %	May indicate some (a) 0-0.5 acidosis in its ab- (b) 0-30 sence	(a) 0-0.5 (b) 0-30	May indicate acidosis in its absence
Mild acidosis. No pronounced symptoms.	No (a) 55–40 (b) 23–17	(a) 27–65 (b) 1600–4000	Good	(a) 35–27 mm. (b) 4.7–3.6%	May indicate more (a) 0.5-0.8 acidosis than is (b) 30-50 present	(a) 0.5-0.8 (b) 30-50	May indicate much more acidosis than is present
Moderate to severe acidosis. Symptons may be applearent.	(a) 40–30 (b) 17–12	(a) 65-100 (b) 4000-6000	Liable <sup>2</sup> to considente able error in (b) 3.6–2.7% either direction	(a) 27–20 mm. (b) 3.6–3.7 %	Good	(a) 0.8-1.1 (b) 50-65	May indicate much more acidosis than is present
Severe acidosis, (a) Below 30 (a) Over 100 Symptoms of (b) Below 12 (b) Over 6000 acid intoxication.	(a) Below 30 (b) Below 12	(a) Over 100 (b) Over 6000	Liable to consider- able error in either direction	Liable to consider- (a) Below 20 mm. able error in (b) Below 2.7% either direction	Good	(a) Over 1.1 (b) Over 65	May indicate much more acidosis than is present

In nephritis neither the NH3+ acid excretion-rate nor the amount of Na3HCO3 required to turn the urine alkaline is at all reliable as a measure of alkali deficit.

Taken by permission of the authors from Peters and Van Slyke. Quantitative Clinical Chemistry.

Measured either in twenty four hour urine or from shorter period calculated to twenty four hour basis.

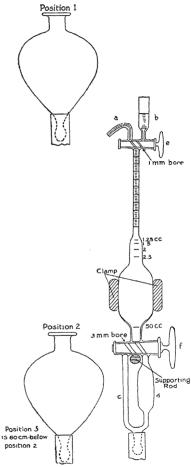
After the theraphorated administration likely to indicate more acideosis is fann is present.

The figures studiated in this column also indicate the does of blearboanet necessary to restore the alkaline reserve to normal from acideosis of the severity indicated by the corresponding plasma COs figures in the first column, according to the results of Palmer and Van Slyke.

tube. If the subject is cooperative, close the nose with a clip, and insert a mouth piece. At the end of a quiet expiration connect the mouth piece with the bag. The patient

rebreathes the air in the bag for 25 seconds, taking 5 inspirations moderately deeper than the average, during this period. After the fifth expiration clamp the bag and replace the mouth piece with a glass tube drawn out to a capillary tip. Insert the tip in a small test tube in which has been put a few cc. of o.or N NaOH solution containing 0.002% phenol red as indicator. Release clamp and press the bag, forcing a current of air through th solution until the color becomes constant. Compare at once with the color of the standard tubes, which must be of the same diameter. These contain M/15 solutions of monobasic and dibasic phosphate (with phenol red in the same concentration) in proportions corresponding to fixed CO2 tensions, from 10, 15, . . . to 45 mm. of Hg. These can be purchased, or prepared according to directions found in special texts. In sealed tubes they keep for several months. If the subject can not cooperate, for the mouth piece substitute a close-fitting rubber mask which is held tightly to the face during the rebreathing period. The procedure is not applicable to patients with circulatory failure or with primary respiratory disturbances. In cases of primary alkali deficit it is the best simple substitute for direct determination of the plasma bicarbonate.

The CO2 combining power (capacity) of the plasma may be determined easily by gas It has about the same significance as the total CO2 content, although they are not numerically equal. (A) Equilibration of the (1) Without stasis collect venous blood with the apparatus shown in Fig. 173, or by means of an all glass syringe containing a few crystals of potassium oxalate, discharging it as soon as possible beneath paraffin oil in a centrifuge tube. Undue loss of CO2 to the air should be avoided until the plasma is Fig. 173.—Van Slyke's plasma bicarin the apparatus. However, a slight degree



bonate apparatus.

of exposure does not affect the result significantly. (2) Centrifuge as soon as possible, and pipette the clear plasma into a separatory funnel, or store in the refrigerator under oil. (3) Attach the funnel (Fig. 174) to a bottle containing moist glass beads and after a normal inspiration, blow one complete expiration through the apparatus, the current passing from beads to funnel. Close both ends of funnel just before finish. (4) To equilibrate, for two minutes rotate funnel so as to distribute plasma in a thin layer.

Analysis.—(1) Fill the entire apparatus (Fig. 173) with mercury, and close stopcock e. (2) Make sure that there are no leaks, and particularly that no air seeps through the pressure tubing when the apparatus is exhausted. It is a great advantage to have the improved form of the apparatus with a Shohl trap fused to the lower end to catch such leakage. (3) Put 1 cc. of CO<sub>2</sub>-free H<sub>2</sub>O in cup b. (4) With leveling bulb in "position 2" and cock f as shown, slightly open cock e, and run 1 cc. of plasma beneath water in cup with the tip of the pipette in the bottom of the cup, so that the plasma flows practically directly into the burette. The pipette should be graduated to deliver between two marks, and not to the tip. (5) Add a drop of octyl alcohol. (6) When capillary above cock e contains only the alcohol run 0.5 cc. of a r-10 dilution of concentrated lactic acid into the apparatus, stopping just when the Hg reaches the 2.5 cc.

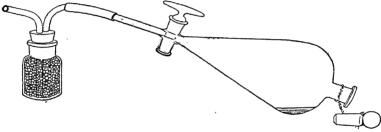


Fig. 174.—Separatory funnel used in saturating blood plasma with earbon dioxide. The bottle contains glass beads. (*Journal Biological Chemistry*, 30, 289, 1917.)

mark. (7) Close cock c and seal with a drop of Hg in cup b. (8) Place leveling bulb in "Position 3," and allow Hg to run down to 50 cc. mark. (9) Close cock f, remove apparatus from clamp, and mix contents by inverting 15 times. (10) Replace in clamp, open cock f, and draw fluid into d, leaving capillary of f full, and allowing no gas to follow. (11) Reverse f, raise leveling bulb, and allow Hg to enter from c and rise slowly and without oscillation. (12) With Hg in leveling bulb higher than that in the apparatus by one-thirteenth of the height of the water column on the mercury, above the cock f, read the gas volume. (13) Calculate volumes per cent of  $CO_2$  by use of the table on page 696, which corrects for temperature, pressure and other factors. (14) Immediately clean apparatus with  $CO_2$ -free water, and let stand filled with water.

The total CO<sub>2</sub> content of the plasma is recommended by Van Slyke as preferable to the CO<sub>2</sub> combining power, and should always be done if the pH is to be determined or calculated. It is determined in the same manner except that the plasma is not equilibrated with CO<sub>2</sub>, but is pipetted directly into the cup from beneath paraffin oil. The table on page 697 is used for correcting the gas volume. The greatest care must be taken not to permit any exposure of the blood to air, (except the brief period it is exposed in the narrow stem of the pipette). The paraffin oil retards but does not prevent diffusion of gases, and during centrifugalization additional precautions must be taken

to prevent some loss of CO<sub>2</sub> (replacing the oil with soft paraffin or filling the tube to the brim with oil, and closing with a rubber vaccine-bottle stopper so as to eliminate air bubbles completely). For one not experienced in this technique the CO<sub>2</sub> combining capacity is the safer procedure.

The CO2 content can also be determined by titration (Van Slyke et al. 1919).

The CO<sub>2</sub> combining power, or content of whole blood may be determined in a similar manner, except that care must be taken that all red cells are washed into the burette, and after the gases have been extracted and the volume read, r cc. of N NaOH is drawn into the burette from the cup under slight negative pressure to absorb the CO<sub>2</sub>. The volume is then read again. The difference between the readings measures the CO<sub>2</sub>. This is corrected in the same manner as for plasma CO<sub>2</sub>, except that the subtraction of the third column is omitted.

Arterial blood may be obtained safely in a similar manner. A very sharp hypodermic needle, about No. 20 gauge, with a  $45^{\circ}$  bevel is inserted through the skin at an angle of  $45^{\circ}$ , and plunged into the radial artery at the point of maximum pulsation. Follow withdrawal of needle by obliterating pressure for 2 minutes and moderate pressure with a gauze compress for 2 to 3 hours. Blood which is practically arterial can be obtained by immersing the forearm in water at  $45^{\circ}$ C. for ten minutes, until the veins are widely dilated, and then drawing blood from a vein on the dorsum of the hand near-the knuckles.

The oxygen combining capacity of the blood, the standard method for determination of haemoglobin, may be measured with this apparatus. (1) 3 cc. of CO-free oxalated blood are aerated in a separatory funnel by rotating it for a few minutes so that the blood is distributed over the walls in a thin film. (2) Wash the apparatus twice with water. (3) Introduce through the cup 6 cc. of water, 0.3 cc. of 1% saponin solution (Merck), and 3 drops of caprylic alcohol. (4) Evacuate, shake thoroughly, and expel the air Repeat until air is all extracted. (5) Expel solution into cup. (6) Stir blood thoroughly, fill 2 cc. pipette, and (7) with the tip of the pipette in the bottom of the cup, under slight negative pressure, allow blood to enter the burette slowly, along with part of the solution. (8) Add o.t cc. of 20% potassium ferricyanide solution (boiled and cooled, to expel air) with the last of the solution. (9) Seal the capillary in cup b with a drop of IIg, evacuate the bulb so far that no Hg can be shaken up into the bulb, and shake for 3 minutes so as to whirl the solution about in the bulb but to avoid violent agitation. (10) Release vacuum, and under slight negative pressure run 1 cc. of gas-free N NaOH into burette, followed by a drop of Hg. (11) Let stand until alkali has drained down from walls of burette (absorbing the CO2), draw the solution into the trap below the chamber, and read the gas volume. (12) Multiply this by the correction factor from column 2, page 697 and subtract 2.1 to correct for N and physically dissolved (). (13) Multiply by 0.746 to get haemoglobin in grams per 100 cc., or by 5.41 to get per cent of haemoglobin in terms of Haldane's standard (18.5 vol. %). Studies in this country indicate that the normal O combining capacity of young adult males is 20.0 vol. %, corresponding to 15.6 grams per 100 cc., according to the usual factor, I Gm. haemoglobin = 1.34 cc. O combining capacity. To get percentage of haemoglobin according to this standard, multiply O combining capacity by 4.78.

The O content of either arterial or venous blood can be determined, with the following slight changes in procedure, but the blood must be protected from any exposure to the air, as above described. The saponin and ferricyanide are conveniently combined in a single solution: 6 Gm. of potassium ferricyanide and 3 Gm. of saponin are dissolved in

Table for Calculation of Carbon Dioxide Combining Power of Plasma<sup>1</sup>

Observed vol. gas × Barometer	760 I	nm. bo	duced t and as l	oicar-	Observed vol. gas × Barometer	760	f CO2 re mm. bo	und ås	bicar-
760	15°	20°	25°	30°	760	150	20°	25°	300
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
I	IO.I	10.9	11.7	12.6	I	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.0	53.8	54.1	54 · I
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	50.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.0	58.8
2	20.7	21.4	22.I	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	01.4	01.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	624	62.6	62.5
6	24.6	25.2	25.8	26.3	0	03.2	03.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	04.2	64.3	64.5	64.3
8	26.5	27.I	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.I	28.7	29. I	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67. r
r	29.4	30.0	30.5	31.0	r	68. r	68. r	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69. r	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33 · 4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	718
6	34.2	34.7	35 . 3	35.6	6	72.0	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.0	73.9	74.0	73.6
8	36.1	36.6	37.2	$37 \cdot 4$	8	74.8	74.8	74.0	74.5
9	37.I	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0 50	38.I	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.6	78.7	78.2
3	41.0	41.4	4I.9	42.I	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	8.4.3	83.8
9	46.8	47.I	47 - 5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.I	48.5	48.6	1.00	86.5	86.2	86.2	85.7

¹ The temperature figures at the heads of columns represent the room temperature at which the samples of plasma are saturated with alveolar carbon dioxide and analyzed. It is assumed that both operations are performed at the same temperature. The figures have been so calculated that, regardless of the room temperature at which saturation and analysis are performed, the table gives the volume (reduced to o°C., 760 mm.) of carbon dioxide that 100 cc. of plasma are capable of binding when saturated at 20° with carbon dioxide at approximately 41 mm. tension. If the figures in the table are multiplied by 0.94 they give, within 1 or 2 per cent., the carbon dioxide bound at 37°C.

# TABLE FOR OTHER BLOOD GAS DETERMINATIONS

Room tempera-	For oxygen determinations	For total CO <sub>2</sub> determinations			
ture, °C.	Correction for reduction to o°C., 760 mm.	Correction for dissolved air	Correction for reduction to o°C., 760 mm.		
1 5	.40.0 $\times \frac{\text{Barometer}}{760}$	0.052	$_{100.2} \times \frac{\text{Barometer}}{760}$		
16	$_{40.4} \times \frac{\text{Barometer}}{760}$	0.051	$99.5 \times \frac{\text{Barometer}}{760}$		
17	$_{40.2}$ $\times \frac{\text{Barometer}}{_{760}}$	0.050	$98.9 \times \frac{\text{Barometer}}{760}$		
18	$45.95 \times \frac{\text{Barometer}}{760}$	0.040	$98.3 \times \frac{\text{Barometer}}{760}$		
19	$45.75 \times \frac{\text{Barometer}}{760}$	0.048	$97.8 \times \frac{\text{Barometer}}{760}$		
	$45.5 \times \frac{\text{Barometer}}{760}$	0.047	$_{97.2} \times \frac{\text{Barometer}}{_{760}}$		
	$45.3 \times \frac{\text{Barometer}}{760}$	0.046	$96.6 \times \frac{\text{Barometer}}{760}$		
	$_{45.05} \times \frac{\text{Barometer}}{760}$	0.045	96.0 $\times \frac{\text{Barometer}}{760}$		
23	$_{44}$ $8_5 \times \frac{\text{Barometer}}{760}$	0.045	$95.4 \times \frac{\text{Barometer}}{760}$		
24	$44.6 \times \frac{\text{Barometer}}{760}$	0.044	$94.8 \times \frac{\text{Barometer}}{760}$		
25	$_{44.4}$ $\times \frac{\text{Barometer}}{760}$	0.043	$_{94.2} \times \frac{\text{Barometer}}{760}$		
26	$_{44.15} \times \frac{\text{Barometer}}{760}$	0.042	93.6 $\times \frac{\text{Barometer}}{760}$		
27	$43.9 \times \frac{\text{Barometer}}{760}$	0.041	93.1 $\times \frac{\text{Barometer}}{760}$		
28	$_{4.5.65} \times \frac{\text{Barometer}}{_{760}}$	0 040	92.4 $\times \frac{\text{Barometer}}{760}$		
20	$43.4 \times \frac{\text{Barometer}}{700}$	0.940	$91.8 \times \frac{\text{Barometer}}{760}$		
30	. Barometer	0.030	$_{91.2} \times ^{\mathrm{Barometer}}$		

I liter of water. At step (3) 10 cc. of this solution with 3 drops of caprylic alcohol are introduced into the burette, and (4) air is extracted. (5) Expel 6 cc. of the solution into the cup, retaining 4 cc. in the burette. (7) Introduce the blood, washing in with 1 cc. of solution. The remaining 5 cc. are discarded. Step (8) is omitted. (12) After multiplying the observed volume by the correction factor in the second column of the table subtract 1.36 vol. % to correct for N. To get O bound by haemoglobin, subtract instead 1.5 vol. % for venous blood, and 1.7 vol. % for arterial blood.

The per cent of O saturation is determined by dividing the observed O content by the O combining capacity. The O unsaturation (in vol. %) is determined by subtracting the O content from the O capacity. Normal arterial blood is 05% saturated.

These analyses can be done more easily and more precisely with the manometric apparatus of Van Slyke, but it is considerably more expensive.

### TESTS FOR BLOOD

Color Tests.—Chemical tests for occult blood in the faeces, urine, etc. are described elsewhere (p. 726 and p. 747). As these depend upon oxidation reactions, although very

sensitive they are not specific and are reliable only when interfering substances can be excluded.

Haemin Crystal Test (Teichmann).—This test is not sensitive, but a positive result is conclusive evidence of the presence of blood pigments. Prepare a solution (stable) of o.r Gm. each of KI, KBr, and KCl in roo cc. glacial acetic acid. Mix a few drops with some of the material on a slide, apply a cover glass, and gently warm until bubbles begin to appear. Then cool slowly, and examine for the characteristic dark-brown crystals.

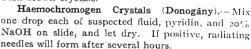




Fig. 175.--Teichmann's haemin crystals. (Todd after Jakob.)

Spectroscopic tests for blood pigment are conclusive if definitely positive, but they require a relatively high concentration of pigment. The degree of concentration influences their appearance, and one should start with a relatively concentrated solution, diluting cautiously until the bands are typical. The small direct-vision (hand) spectroscope usually suffices, but for such procedures as detecting and differentiating between methaemoglobin and sulphaemoglobin in clinical conditions a more powerful instrument is often required. A wave-length scale is a convenient attachment. Daylight or strong artificial light (such as the "daylite" lamp) is used. Have solution in a small test tube or, preferably, a flat cell with a thickness of about 1 cm. Before use, focus Frauenhofer's lines sharply. The solutions must be clear; filter or centrifuge if necessary.

Reducing agents, such as ammonium sulphide or Stokes' solution, are employed. These must be fresh, and, before use, the sulphide must be warmed to about 50°C. To prepare Stokes' solution, dissolve 3 Gm. FeSO<sub>4</sub> in cold H<sub>2</sub>O, add cold aqueous solution of 2 Gm. tartaric acid, and make up to 100 cc. Immediately before use, add strong, NH<sub>4</sub>OH until precipitate first formed is dissolved.

Material that is uncontaminated, relatively fresh and in relatively concentrated aqueous solution may give any or all of the upper three spectra, a few drops of reducer changing the first or third to the second (Fig. 176).

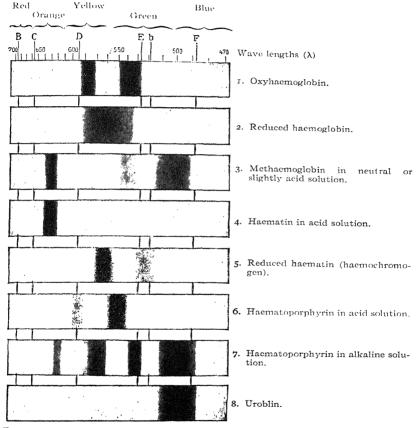


Fig. 176.—The most important clinical spectra. (Modified from Monographic Medicine. D. Appleton & Co., New York.)

For old dried blood stains the following procedures may be tried:

- 1. Dissolve the suspected stain in 1-2 cc. of 10% NaOH, heat almost to boiling, cool, and add a few drops of reducer. Examination shows Spectrum 5.
- 2. Old and relatively uncontaminated stains are discourse WON all warmed to 40°C. Examination shows cyanhaematin

bling Spectrum 2). Reduction with ammonium sulphide gives cyanhaemochromogen (similar to Spectrum 5). In cyanide poisoning, blood pigment in walls or lumen of stomach shows cyanhaematin.

- 3. With very old and relatively uncontaminated material, if insoluble in KCN solution, a small fragment is mixed with a few drops concentrated H<sub>2</sub>SO<sub>4</sub>, and crushed and rubbed between two glass slides. Examination shows Spectrum 6.
- 4. If very old and much mixed with other material, pulverize, mix with a few cubic centimeters concentrated H<sub>2</sub>SO<sub>4</sub>, and let stand twenty-four hours. Filter through glass wool or asbestos, and pour filtrate into 10 times its volume of distilled water. Wash the brown precipitate several times, and dry. Dissolve in a mixture of equal parts of absolute alcohol and strong NH<sub>4</sub>OH, and filter. Examination shows Spectrum 7, which, upon acidification, will change to a faint Spectrum 6.

It is better, however, especially with much contamination, to prepare an ethereal, acid extract. This not only frees it from contaminating substances but makes possible a concentration of the pigment. After having ground the material thoroughly with water, if it is not already in liquid form, mix gently with an equal volume of neutral ether. Reject ether extract, and, to ro cc. of residue, add 3 to 5 cc. of glacial acetic acid. Shake thoroughly with an equal volume of ether. If the ether does not separate readily, mix gently with a few drops of alcohol. Remove ethereal extract, and evaporate it to a small bulk for use in tests. Examination will show spectrum of acid haematin, which, however, in ethereal solution, resembles Spectrum 3 more than 4. Make alkaline with strong NH4OH, cool, mix well, let separate, and add a few drops of reducer to the ammoniacal extract. Examination shows Spectrum 5.

Donogány's method increases the delicacy of the spectroscopic test, and is also a color test. Dissolve the pigment with 20% NaOH, add fresh pyridin and, if necessary, fresh ammonium sulphide. Filter. The filtrate will be more or less orange-red according to blood content, and will show Spectrum 5 (Fig. 176).

The spectrum of carbon monoxide haemoglobin resembles that of oxyhaemoglobin except that the bands are shifted slightly toward the violet, and are closer together. Addition of reducing agents does not change it to reduced haemoglobin (demonstrable only in concentrations of at least 30 vol. %). Chemical tests are more sensitive (see p. 701). To detect and measure small quantities gasometric determinations are necessary.

Demonstration of methaemoglobin is important in poisoning with certain drugs such as acetanilid. Sulphaemoglobin, long confused with the preceding and often present with it, has a similar band which persists after the addition of reducing agents. It is abundant in cases of "enterogenous cyanosis." The spectrum of acid haematin which also has a similar band near C is changed by reducing agents to Spectrum 5.

## Color Tests for Carbon Monoxide in Blood

- 1. Katayama's test.—Put 5 drops of suspected blood and 5 drops of normal blood in each of two test tubes. Dilute each to 10 cc. with water, add 5 drops of fresh yellow ammonium sulphide solution, and acidify slightly with acetic acid. Normal blood turns a dirty greenish brown color; blood containing carbon monoxide retains more or less of a reddish tint.
- 2. Haldane's dilution test.—Dilute 1 cc. of suspected blood to 100 cc. with water, and similarly 1 cc. of normal blood as a control. Compare the colors against a white back-

ground. Blood containing CO has a purplish pink tint as compared with the yellowish red of normal blood.

3. Sayers and Yant's tannic acid method gives an approximate quantitative estimate of the CO present. In a small test tube dilute 0.1 cc. of suspected blood with 0.4 cc. of dilute ammonia (1 cc. of strong ammonia per liter). Mix and add 1.5 cc. of freshly prepared 1% tannic acid solution. Let stand over night and compare the color either with permanent standards or standards prepared simultaneously as follows: Bubble CO or illuminating gas through 1 cc. of oxalated blood until saturated. Dilute this and also 1 cc. of normal blood with 4 cc. of dilute ammonia. Make up a series of mixtures of these laked bloods (in 0.5 cc. volume) corresponding to 10, 20, 30, 40, 50, and 60% saturation. Add 1.5 cc. of tannic acid solution to each.

In none of these tests are the color differences well marked if the CO saturation is under 20%. In acute poisoning the saturation usually ranges from 25% to 60% or more, but it falls quickly after the patient is removed from the source of poisoning (30% to 50% per hour in air, about four times as fast in 0.5% O and 5% CO<sub>2</sub>).

Diastase.—This may be determined by Winslow's method (page 731), substituting 1 cc. of serum or whole blood for urine. Normal figures are from 8 to 64 units. The quantity is increased in diabetes mellitus, in nephritis with renal insufficiency, and markedly in severe pancreatic disease.

# CHAPTER XXXIV

# BLOOD CULTURES

THE demonstration of bacteria in the blood is of great importance in the diagnosis and prognosis of various infections. Only very rarely are the ordinary bacteria sufficiently numerous in the blood to be seen in smears, but it is usually a simple procedure to obtain them by culture.

A transient bacteriaemia occurs frequently in many of the acute infectious diseases, especially at the onset. It is especially common in the typhoid group of infections, the pneumonias, undulant fever, and may occur in cerebrospinal meningitis and other infections. In these cases the presence of a few organisms in the blood is of great diagnostic importance, but is without special significance so far as prognosis is concerned. On the other hand, if they are present in large numbers, especially if they persist, and their number increases, the prognosis becomes grave. In some of these cases, however, such as meningococcus or pneumococcus sepsis, serum treatment may be effective, at least in removing organisms from the blood stream.

From a focal infection anywhere in the body organisms of many kinds may invade the blood stream. As a rule the bactericidal power of the blood is sufficiently great to kill these bacteria, so that the blood remains sterile. Occasionally, however, owing to a diminished resistance or to the high virulence of the infecting organism, the bacteria survive, and appear in the blood in large and increasing numbers. This increase is due, not to multiplication in the blood stream, but to greater invasion from the focus. Such a condition is known as septicaemia or sepsis. If an indvidual lives long enough, secondary suppurating foci may occur, and the condition is termed pyaemia. Bacteria found in the blood in these conditions have a much more serious prognostic significance.

Certain organisms of relatively low virulence may grow and multiply on the heart valves, and some escape more or less constantly into the circulation. There may be periods in the chronic cases in which blood cultures are sterile, and repeated tests may be necessary to demonstrate them. Such an endocarditis is most often due to the Streptococcus viridans. Haemolytic streptococci cause a rapidly fatal, ulcerative endocarditis.

In the interpretation of the results of blood culture the clinical picture must be considered. The significance of pneumococci in a blood culture is not the same in an early case of lobar pneumonia, in a chronic endocarditis, and in an acute mastoid infection, for example.

Although blood invasion by the staphylococcus may occur, this organism is regularly present on the skin; and blood cultures yielding a few colonies, especially of Staphylococ-

cus albus, should be viewed with suspicion, and the results corroborated before accepting them as significant. Diphtheroid bacilli are also commonly present on the skin, and various bacteria from the air may contaminate a culture. However, if the blood is cultured immediately, and if part of it is planted in solid media (blood agar plates), it is usually easy to recognize a contamination as such, should one occur.

In taking blood for culture it is desirable to select a time when the temperature is high or rising. The methods for making cultures vary in details with the type of organism suspected, but the following serves as a good routine.

Technique.—The blood is withdrawn from some prominent vein, preferably the median basilic vein of the arm, by means of a sterile 10 or 20 cc. syringe. If Luer syringes are used they may be kept sterilized, together with a needle, in large plugged tubes in the hot air sterilizer, or boiled for from 5 to 10 minutes just before obtaining the blood. If a syringe is not available, the blood may be obtained by the device shown in Fig. 201, with care to plug the aspirating arm with cotton before sterilizing to prevent saliva from getting into the blood. Large Keidel tubes containing sodium citrate, alone or combined with glucose broth, are convenient if only an occasional culture is made.

The skin over the vein should be scrubbed thoroughly with soap and water, and painted with tincture of iodine. A tourniquet is applied to distend the vein during the puncture. Ten or lifteen cc. of blood are withdrawn, and the tourniquet is removed before drawing out the needle. The iodine is then washed off with alcohol, and the puncture covered with sterile gauze or cotton. The blood is added to a 50 cc. flask containing 5 cc. of 1% sterile sodium citrate solution in physiological saline. After removing the stopper the mouth of the flask should be flamed before introducing the blood. The citrate prevents clotting until the blood can be inoculated into the various media. The culture can be made at the bedside when feasible, but the use of the citrate flasks is generally more convenient. The inoculations should be made as soon as possible. The blood can be transferred to the media with a sterile cotton-plugged rocc, pipette, or, with a little experience in judging quantities, poured into the following media.

- a. One or two cc. in each of 2 or more tubes of melted agar cooled to 42°C. Mix and pour into sterile Petri dishes.
  - b. One or two cc. in 2 or more tubes of dextrose brain broth.
  - c. Five cc. in a flask containing 100 cc. of glucose broth.

The remainder of the citrated blood may be incubated as a control. The cultures should be examined daily for evidences of growth. Colonies developing on the plates should be counted and subcultured for identification. If growth appears in the broth, transfers should be made and smears examined. After 72 hours subculture the broth on a blood agar slant, even though no growth is evident. The original cultures should be kept for at least 2 weeks, and preferably a month, since some bacteria grow slowly when first isolated.

Special modifications of this method are used for some cultures. If anaerobic organisms such as anaerobic streptococci are suspected, part of the culture must be incubated under anaerobic conditions. In large laboratories a McIntosh and Fildes jar can be used, or some modification of the pyrogallic acid and sodium hydroxide method. (See section on anaerobic methods.) Fairly satisfactory anaerobiosis can be obtained by inoculating the blood into deep narrow tubes of glucose agar or glucose broth which have been previously heated to remove oxygen, cooled, and covered with sterile vaseline. Some workers make, as a routine, deep tubes of glucose ascitic fluid agar without

vaseline in order to obtain varying degrees of oxygen tension in the culture. See page 832. Anaerobic bacilli associated with wound infections seldom invade the blood stream, although the gas bacillus has been obtained in blood culture.

When B. abortus is suspected, ten to fifteen cc. of blood are distributed in varying quantities into small flasks containing 30 cc. of meat infusion broth pH 7.5. Several agar slants are inoculated by flooding their surfaces with blood. Incubate at 37°C. in an atmosphere with a CO<sub>2</sub>-tension of 10%. Since the organisms grow slowly, the cultures should be examined and subcultured on glucose agar slants from the 4th to the 14th day. Ten per cent CO<sub>2</sub> tension may be produced by any of the methods described (see p. 833).

Cecil uses the following method for obtaining streptococci from the blood. Twenty cc. of blood are allowed to clot in two tubes in the ice box over night. Each clot is then broken up with a sterile glass rod in a flask containing 50 cc. of beef heart infusion broth pH 7.5, and incubated at 37°C. for a month. At 5 day intervals subcultures are made on blood agar and in blood broth. The risk of contamination in this method is great, and the manipulations should be made under a hood. With this method, moreover, one is not able to estimate the number of organisms present in the blood, and the results obtained must be accepted with reservation. It is quite probable that an occasional organism may enter the blood stream in conditions unrelated to streptococcus infection, and may be obtained accidently in cultures when large quantities of blood are inoculated into fluid media. Lightman and Gross (1932) in a large series of blood cultures obtained alpha and gamma streptococci in such conditions as pyelitis, pernicious anaemia, leukaemia and meningococcus meningitis, in about the same frequency (6%) as in rheumatic fever and rheumatoid arthritis.

For culturing typhoid or paratyphoid bacilli some of the blood may be added to tubes containing sterile ox bile, or bile to which 10% of glycerin and 2% of peptone has been added. Daily transfers are made to agar or broth tubes for identification. Since the typhoid bacilli flourish in bile more readily than do most organisms, it is often possible to obtain them in blood obtained from the finger with a Wright tube by dropping the clot into a tube of bile (clot culture). Blood obtained in this way is apt to be contaminated, however, and it is better to transfer from the bile culture directly to Endo or other selective media.

The tubercle bacillus rarely occurs in the blood except in miliary tuberculosis. In these cases growth can be obtained by laking the blood with sterile distilled water and planting the centrifugalized sediment on suitable solid media. Since growth is slow, the moisture in the medium must be conserved by sealing the tubes with paratiin or vaseline after a few days.

Animal inoculation is less used for obtaining bacteria from the blood than from contaminated material, but it is essential in some cases. In tularaemia the organisms have been obtained from the blood in the first week of the disease by inoculating rabbits or guinea pigs intraperitoneally with from 2 to 4 cc.

Anthrax and plague bacilli can be demonstrated by the inoculation of mice or guinea pigs. Tubercle bacilli are easily found by guinea pig inoculation. The spirochaetes of Weil's disease may be seen in direct smears from the blood, but are difficult to cultivate on artificial media. Their presence, however, can be demonstrated easily by guinea pig inoculation. When trypanosomes cannot be found in smears animals should be inoculated.

In animal inoculation from r to 5 cc. of blood are injected intraperitoneally or subcutaneously. After the animal's death, cultures can be made from the heart's blood, liver, and spleen, and smears from these organs usually show the organisms. Characteristic lesions may be found in some cases.

The presence of some of the *rickettsias* and *filtrable viruses* in human blood has been demonstrated by the production of lesions in animals, and by the development of an infection from which, after recovery, they can be proved to be immune. This method is of great use in the diagnosis as well as in the investigation of diseases due to filtrable viruses.

Animal parasites found in blood.—The examination of the blood for the parasites of malaria, filariases, kala-azar and spirochaetal fevers has been discussed under their respective headings.

With trypanosomes from human trypanosomiasis, smears from gland juice or cerebrospinal fluid seem more satisfactory to examine than blood smears unless the blood is taken in 5 to 10-cc. quantities and centrifuged in sodium citrate-salt solution.

A method in the diagnosis of trichinosis is to take 5 to 10 cc. of blood from a vein at the time of the migration of the embryos to the muscles (ten to twenty days). The blood is forced out into a centrifuge tube containing 3 volumes of 3% acetic acid, and the sediment examined for trichina larvae.

# CHAPTER XXXV

# EXAMINATION OF THE URINE

For routine purposes it usually suffices to examine two single voidings; one, obtained about two hours after the principal meal, which is most likely to show sugar and albumin, if present; and a second, obtained on rising in the morning, which gives a check on the concentrating power of the kidney. A note should always be made regarding the color, turbidity, specific gravity, reaction, microscopic examination of the sediment, and chemical tests for albumin, sugar and acetone. For quantitative chemical studies it is necessary to collect the total urine for 24 hours, keeping it on ice, or using a preservative (preferably 2% toluol) to prevent bacterial decomposition; or for shorter fixed periods, under standard conditions.

Color.—Normally urine varies from pale straw to deep amber, according to its concentration, owing to the pigment urochrome. Rarely, it is pinkish (urocrythrin). In jaundice (bilirubin) it shows varying tints from orange-yellow to deep brown, and a yellowish foam when shaken. Urobilin in large amount also gives it a brownish tinge, but a colorless foam. In haemoglobinuria it shows a clear reddish color. In haematuria it varies from a smokey tinge to opaque bright red or reddish brown. A black tinge suggests melanin, which may be found in melanotic sarcoma, or in the rare constitutional anomaly, alcaptonuria. Unusual tints (of yellow, brown, red, green or blue) may follow the administration of a great variety of drugs.

Turbidity.—Gross turbidity in an old specimen of urine is usually due to the growth of contaminating bacteria, to amorphous urates (if acid), or to carbonates and phosphates (if alkaline). In freshly voided acid specimens it is usually due to infection (pus cells and bacteria), but in alkaline specimens (shortly after a meal) it is often due to carbonates and phosphates which dissolve after the addition of a few drops of dilute acetic acid. A faint opalescence may be due to bacteriuria (without pus cells), as is frequent in typhoid fever. In *chyluria* the urine is opaque and milky white. It follows rupture of a lymph vessel into the urinary passages. It is usually (but not always) due to filariasis. The embryos may be present in the urine.

Specific Gravity.—This is significant only when the specimen is collected under standard conditions. If no fluid is taken during the evening and the bladder is emptied on retiring, the early morning voiding should have a sp. gr. of 1.020 or higher. A very low sp. gr. is usually due to excessive fluid intake, and should be viewed with suspicion in the case of a candidate appearing for physical examination, since pathological constituents often escape detection in the resulting dilution. (See tests for renal function.) A low sp. gr. in the 24 hour specimen may be due simply to a restricted diet, a low salt intake, or copious water drinking.

Reaction.—For most purposes tests with litmus paper suffice. Normally the reaction is acid, except that a specimen shortly after a meal is usually alkaline ("alkaline tide"). Occasionally (in conditions associated with acidosis), it is desirable to determine the acidity of the urine more precisely, either by estimating the pII, or by measuring the titrable acidity (see p. 730).

Urinary Sediments.—For the examination of organized sediments, the specimen should be as fresh as possible. If it is not to be examined immediately, it should be kept cold, or a little formalin should be added. Red cells and casts disintegrate rapidly in warm specimens, particularly if dilute and alkaline.

Sediment is best concentrated by centrifugalizing 15 cc. of urine at 1500 revolutions per minute for 5 minutes (not longer), decanting the supernatant urine, and suspending the sediment in the few drops of urine that flow back from the walls of the tube. Take up some sediment with a capillary pipette and mount on a slide one drop of such size that when covered with a 22 mm. square cover slip it does not flood out from under the cover slip. Examine with dim illumination under the low power, particularly for casts.

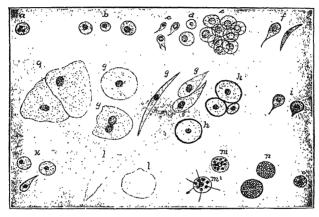


Fig. 177.—Epithelium from different areas of the urinary tract. a, Leukocyte (for comparison); b, renal cells; c, superficial polvic cells; d, deep polvic cells; e, cells from calices; f, cells from ureter; g, g, g, g, g, squamous epithelium from the bladder; h, h, neck-of-bladder cells; i, epithelium from prostatic urethra; k, urethral cells; l, l, scaly epithelium; m, m', cells from seminal passages; n, compound-granule cells; o, fatty renal cell. (Ogden.)

and identify individual cells present with the high dry objective. A drop of Gram's iodine solution may be added to stain the cells.

In order to secure a uniform degree of concentration, in the laboratories of the U.S. Naval Medical School it is customary to resuspend the sediment in exactly 2 cc. of urine. This facilitates the quick determination of an excessive number of red cells or pus cells without recourse to elaborate counting procedures.

Epithelial Cells.—Moderate numbers of epithelial cells are constantly present in normal urine. Large cells of the squamous type arise from the bladder or vagina. Smaller cuboidal or spherical cells which are attached to casts or which are filled with coarse refractile granules come from the kidney. It is practically impossible to determine the source of other types of epithelial cells with certainty from their appearance. Sheets of more or less round or caudate epithelial cells are suggestive of pyelitis. For morphology of cells from different locations see Fig. 177.

Cells with highly refractile lipoid granules (doubly refractile under a polarizing microscope) are common in the "nephroses."

Renal cells containing haemosiderin granules are common in haemochromatosis and occur in the active stage of various haemolytic anaemias. Rous' method (1918) may be used to demonstrate them. (1) Centrifugalize a freshly voided specimen of urine and decant. (2) Examine a little of the sediment for brownish granules (intracellular and extracellular). (3) Resuspend the rest of the sediment in a freshly-mixed solution containing equal parts of 2% potassium ferrocyanide and 1% HCl. (4) After ten minutes centrifugalize and examine the sediment for blue granules (high dry objective). A normal urine should be treated in the same way as a control.

Leukocytes.—To differentiate a leukocyte from an epithelial cell it is necessary to see the lobes of the nucleus clearly. This is often impossible unless the preparation is stained (as with Gram's iodine solution), or cleared with a drop of dilute acetic acid, which dissolves the cell granules, and brings out the outline of the nucleus distinctly.

A note should be made of the approximate number per high power field. A convenient method of recording is:  $+^1$ , (5 to 20 cells);  $+^2$ , (20 to 50 cells);  $+^3$ , (50 to 34 filled);  $+^4$ , (packed field). If abundant, note volume of packed cells in the centrifuge tube. Precise counts are rarely worth while.

Pus cells in a voided specimen of urine from a female may be of no significance, because of the frequent contamination with vaginal discharge. In a catheterized specimen or in male urine, more than an infrequent leukocyte is pathological. (Maximum of 3 per high power fluid, if concentrated under standard conditions.) In acute infections of the urinary passages large numbers of pus cells are present, often in clumps. Pus cells are frequently found in acute and chronic nephritis. A renal origin can be determined only when they are attached to casts.

In males, the use of two sedimentation glasses will differentiate pus from the urethra from pus coming from the bladder or pelvis of the kidney. If the urine in the first glass alone is turbid, it indicates urethral pus. The presence of pus cells attached to macroscopic shreds of mucus suggests a chronic prostatitis and posterior urethritis, which may, but need not, be gonorrhoeal. It is impossible to differentiate between cystitis and pyelitis by examination of urine voided, or catheterized from the bladder. Differentiation must be made clinically or by ureteral catheterization.

In every male with pus cells (in any number) in the urinary sediment, one should examine the prostatic secretion obtained as described on p. 45. A drop of 10% acetic acid (and a trace of methylene blue) should be mixed with a little secretion, and a drop examined ( $\frac{1}{10}$ 6 inch objective). Normal secretion contains epithelial cells, lecitind droplets, "amyloid bodies," and an occasional pus cell, up to about 6 per high power field. More than ten cells per field is pathological—particularly if some clumps are present.

Erythrocytes.—For the demonstration of red cells in urine, fresh specimens are particularly important. This condition (haematuria) must be distinguished sharply from haemoglobinuria, in which dissolved blood pigment (but not intact cells) is present. The red cells may be morphologically intact, or be swollen, and often laked, so that only shadows remain, or shrunken, crenated, and distorted in varying degrees, and occasionally fragmented. They must be distinguished from vegetable spores, yeast cells, refractile droplets and certain crystals, which sometimes resemble them. Their usually uniform diameter of 6 to  $8\mu$  suffices. In case of doubt, a test for occult blood should be applied to the sediment, but it must be remembered that red cells can be

demonstrated microscopically with ease in urine which contains too little blood to give a positive benzidine test.

The approximate number of cells per high power field should be recorded, and, if grossly bloody, the volume of packed sediment: +1, 3-8 cells; +2, 8-30 cells; +3, 4 filled field; +4, packed field.

Addis Counts.—A few red cells are present in the urine of many normal individuals. Addis (1020) kept subjects on a dry diet for a day, allowing the usual foods but no liquids. He then collected the urine for 12 hours (8 P.M. to 8 A.M.) during which fluids were still withheld, concentrated the sediment from 12.5 cc., and resuspended it in 0.5 cc. of urine. In such concentrated acid urine the structures are well preserved. He made continuously of the red cells, etc., by use of a haemacytometer, and calculated the total excretion for the twelve hour period. He found red cells in 40 of 64 normal medical students, the total excretion ranging from 0 to 425,000 cells (820 per cc.). Counts of well over 1,000,000 he regards as pathological, and in the absence of other sources of bleeding, as evidence of active nephritis. The procedure is too time consuming to be practicable for routine use but appears to be valuable in special cases in the detection of mild or latent nephritis. (Haematuria occurs only intermittently in these cases.) Adequate information can usually be obtained by examining in the ordinary way a fresh preparation of the sediment collected by Addis' method.

In women, contamination with vaginal and even rectal bleeding must be excluded. Haematuria is most often met with in the following conditions: (1) Trauma. (2) Acute infections of any part of the urinary passages, including renal tuberculosis. (3) Benign or malignant growths, particularly hypernephroma and vesical polyps. (4) Benign prostatic hypertrophy. (5) Renal, ureteral or vesical calculi. (6) Kinks or strictures of the ureter. (7) Acute nephritis or acute exacerabations of a chronic nephritis, occasionally in arterioscierotic nephritis. Blood cells in casts are always from the kidney. (8) Poisoning with certain drugs, such as bichloride of mercury, phenol, turpentine, cantharides, oil of sandalwood, methanamine. (9) Chronic passive congestion. (10) Renal infarctions, and bacterial emboli in sepsis. (11) Severe infections, such as small pox, plague, yellow fever. (12) Certain tropical parasitic infestations, as bilharziasis and filariasis. (13) General systemic diseases with a tendency to bleeding, such as purpura, and leukaemia. (14) In some cases ("idiopathic haematuria") in which no cause can be found.

Haemoglobinuria occurs most frequently in paroxysmal haemoglobinuria, black water fever, and after reactions to transfusions of incompatible blood.

Casts.—Casts are formed by coagulation of albuminous material in the lumina of the tubules of the kidney. Their shape and size correspond to that of the tubules in which they are formed. They should be searched for with the ½5 inch objective, with the light well dimmed. They are cylindrical, fairly uniform in diameter, and have rounded ends. Hyaline casts show no internal structure. Finely granular casts are similar, but show tine granulation. Both will be overlooked with bright illumination. They indicate some irritation or inflammation of the kidney. In general, they occur in conditions associated with albuminuria, and have about the same significance (see p. 716). Cylindroids are similar to hyaline casts except that their ends are elongated and tapering. They have the same significance. They must be distinguished sharply from broad, flat, strap-shaped strands of mucus, which are not renal in origin. Hyaline and finely granular casts are practically always present if casts of other types are present, and usually greatly outnumber the others. An approximate estimate of the

number should be made: if sparse, the number in an entire coverslip preparation; if more numerous, the number per low power field:  $(+^1, 1 \text{ to } 5; +^2, 5 \text{ to } 10; +^3, 10 \text{ to } 30; +^4, packed)$ . The number of other types of casts, if present, should also be noted

A lew myanne and finely granular casts can be found in many normal individuals. Addis found them in 45 of 74 normal students. The number excreted in a twelve hour period ranged from zero to 4270 (80 per cc.).

Waxy casts are similar to hyaline casts in showing little or no internal structure, but are more highly refractile, denser, more opaque looking, often show slits or cracks and jagged broken ends. Transitions to hyaline casts occur. They occur most often in advanced chronic nephritis. They may give a positive amyloid reaction.

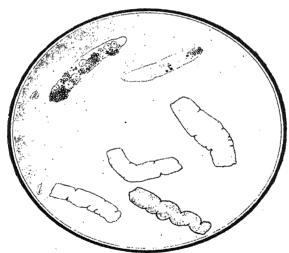


Fig. 178.—Fatty and waxy casts. a, Fatty casts; b, waxy casts. (Greene.)

Coarsely granular casts usually indicate nephritis. Renal epithelial cells, red cells or pus cells may be attached to casts of any type, or may make up the bulk of the cast. They indicate an active nephritis and usually an acute process. The epithelial cells often show granular degeneration, and the coarsely granular and fatty casts contain the products of cellular degeneration. Very broad casts (renal failure casts) are character istic of the advanced stage of renal disease.

Coarsely granular casts may be simulated by incrustations of precipitate about thick strands of mucus in decomposing specimens.

Unorganized Sediments.—The types and quantity of crystalline precipitate depend mainly upon the reaction and degree of concentration of the urine. An excess of any type of crystal is not indicative of a disturbance of metabolism. With few exceptions they are of no practical significance.

The following are found in acid wrines: (1) Sodium and potassium acid urate: amorphous, yellowish-red granules which dissolve on heating, or on adding alkali. (2) Uricacid: yellowish crystals, usually of a whetstone shape, often clustered, rarely in flat plates; soluble in alkali, but not on heating. (3) Calcium oxalate: small, highly refractile, octahedral crystals ("envelope crystals"), or rarely dumb-bell shaped or spherical. They are increased with a diet rich in oxalates (asparagus, spinach, tomatoes, rhubarb). The presence of clusters of calcium oxalate or uric acid crystals in freshly voided urine suggests that conditions in the urinary passages are favorable for calculus formation, but does not prove their presence. (4) Rarely, cholesterol, cystin, tyrosin, leucin, xanthin, haematoidin, biliverdin, indigo, melanin, creatinine, hippuric acid, sodium biurate, calcium sulphate, neutral calcium phosphate.

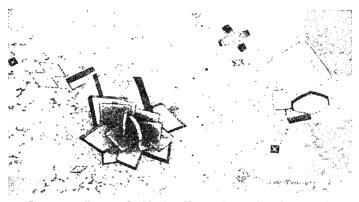


Fig. 179.—Common sediments of acid urine: Uric acid crystals, calcium oxalate crystals, and amorphous urates. ×150. (J. C. Todd "Clinical Diagnosis.")

In alkaline urine one finds: (1) Triple phosphate (NII<sub>4</sub>MgPO<sub>4</sub>.6H<sub>2</sub>O) crystals, in coffin-lid or fern-like form, easily soluble in acetic acid. (2) Ammonium biurate crystals, yellow, thorn-apple in shape, soluble in acetic acid. (3) Calcium phosphate, in slender radiating crystals or flat plates. (4) Magnesium phosphate (rarely). (5) Tricalcium phosphate [Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>], Magnesium phosphate [Mg<sub>2</sub>(PO<sub>4</sub>)<sub>3</sub>], and calcium carbonate as fine amorphous precipitate, easily soluble in acetic acid, the latter with effervescence. They are the usual cause of turbidity in freshly voided, alkaline normal urine. They are often precipitated by heating an alkaline urine.

The presence of numerous ammonium biurate and triple phosphate crystals in *freshly* voided urine indicates bacterial decomposition within the bladder. It occurs only where there is stasis, as in urethral obstruction, or paralysis of the bladder musculature, and is usually associated with a secondary acute cystitis.

Cystin crystals occur in the urine (cystinuria) in a rare constitutional anomaly which results in an inability of the body to break down protein completely in the normal manner, and which is characterized clinically by a marked tendency to form renal calculi. They appear as transparent, hexagonal plates, (sometimes only after con-

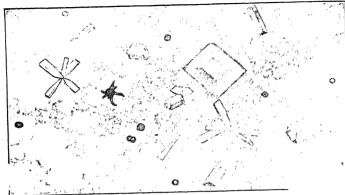


Fig. 180.—Common sediments of alkaline urine: Triple phosphate crystals, calcium phosphate crystals, ammonium urate crystals, and amorphous phosphates. X150. (J. C. Todd "Clinical Diagnosis.")

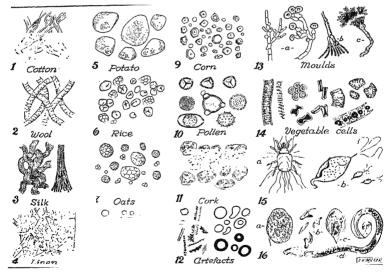


FIG. 181.—Fibers, starch granules, etc., which may be found in urine acdiment. No. 12 gives appearance under microscope of scratches on old used glass slides. No. 15(a), Tyroglyphus longior, a mite. No. 15(b), Trichomonas vaginalis. No. 16 (a), Egg of Dioctophyme; (b) Echinococcus hooklets; (c), Schistosoma egg; and (d), Wuchereria bancrofti embryo.

centration of the urine). They can be distinguished from uric acid and other similar crystals by the fact that they dissolve in HCl but not in acetic acid; that they dissolve in ammonia, and recrystalize when the ammonia is driven off by gentle boiling; and that when dried and heated they char, and give off an odor of burning wool.

Leucin and tyrosin are excreted in cases showing marked destruction of liver tissue, (acute yellow atrophy), but crystals are rarely found unless the urine is concentrated. Urine should be freed from albumin and filtered (preferably with basic lead acetate, removing excess of lead with H<sub>2</sub>S). It is concentrated on a waterbath to a syrup; urea is removed by several extractions with alcohol; and the residue is extracted with hot, dilute, ammoniacal alcohol. The latter is filtered, concentrated, and allowed to stand. Leucin appears as small, spherical, yellowish crystals which show radial and concentric striations, and which dissolve in HCl and boiling glacial acetic acid. Tyrosin appears as yellowish or blackish, fine needles arranged in spherical clusters or sheaves. They can be identified by their solubility in HCl and in ammonia (but not in acetic acid), and by the fact that they give a green color when boiled with Mörner's reagent (1% formalin in 55% H<sub>2</sub>SO<sub>4</sub>).

Extraneous particles.—In examining urinary sediments it is important to be familiar with the various textile fibers and starch grains which are so frequently present, the fibers coming from the clothing and the starch grains from dusting powders. Wool fiber fragments show bark or scale-like imbrications and are round. Cotton fibers are flattened and twisted, whereas linen ones show a striated flattened fiber with frayed segments as of a cane stalk. Silk shows a glass-like tube with mashed-in ends.

Corn and rice grains are the most common of the starch grains and their nature is immediately disclosed by their blue color when mounted in iodine.

### Analysis of Urinary Calculi

(Klopstock and Kowarski.) There are four principal types of urinary calculi:

- (1) Uric acid or urates. Acid sodium urate is more common than ammonium urate.
- (2) Phosphates of calcium and magnesium and calcium carbonate. (3) Calcium oxalate. (4) Cystin and xanthin (rare). (5) Mixed calculi with layers of different composition.

To some extent calculi may be differentiated by their color, the character of the surface and their consistency.

Color.—Urate stones are yellow to dark-brownish-red; phosphate, whitish, grey or greyish-yellow; oxalate, chiefly brownish-red to black, although the smaller ones may be white or grey; cystin, pale yellow; and xanthin, bright brown.

Surface appearance.—Oxalate calculi are rough or tuberculated (mulberry stones); urate calculi are slightly rough. Phosphate calculi have a smooth or sandy surface; cystin and xanthin stones are usually smooth.

Consistence.—Cystin and phosphate stones are the softest; the former are wax-like, the latter earthy or chalky and somewhat brittle. Urate stones are much harder, and oxalate calculi are the hardest.

## Chemical Examination

The larger stones should be sawed into two pieces, the cut surfaces smoothed and washed with water. If it is possible to distinguish a distinct nucleus surrounded by concentric layers, split off the successive layers with a knife, and examine the nucleus and

each layer separately. If the stone appears homogeneous, crush it and grind it up to a fine powder in a mortar.

Transfer a portion of the powder to a piece of platinum foil and heat.

A. If the powder burns up entirely or leaves only a slight residue it is composed of organic materials (uric acid, urates, cystin, xanthin).

Urate and xanthin stones burn without a flame and give off an odor of prussic acid. Cystin stones burn with a bluish flame and give an odor of sulphuric acid. To differentiate:

- r. Moisten a second portion of powder with nitric acid and evaporate to dryness. If the addition of a drop of ammonia to part of this dried residue gives a purple-red color, and if a drop of sodium hydroxide gives a blue-violet color, the stone contains uric acid or urates (murexide test).
- 2. If the addition to the original powder of a drop of sodium hydroxide yields ammonia, the stone contains ammonium urate. If it does not do this and burns completely with a glow, it is pure uric acid. Other urates yield a slight residue.
- 3. If the addition of ammonia to the dried powder gives no color, but if sodium hydroxide gives a bright red color, the stone is of xanthin.
- 4. Cystin stones give no color with either. They dissolve easily in ammonia and separate from the solution as hexagonal plates when the ammonia evaporates.
- B. If the specimen does not burn appreciably, or if it only blackens and (after glowing) leaves a considerable residue, it is composed largely of phosphates, carbonates and oxalates.
- 1. Dissolve the residue (or some of the original powder) in hot dilute IICl. (Any organic matrix or uric acid present will not dissolve.) Cool and clear by centrifugalization or filtration and test the insoluble portion for uric acid by the murexide test.
- 2. Dilute the filtrate with a little water and add ammonia until strongly alkaline. The formation of a precipitate may be due to (a) earthy phosphates (calcium and magnesium phosphates, or triple phosphate—ammonium magnesium phosphate) or (b) calcium oxalate.
- 3. Remove the precipitate by centrifugalization, and to it add acetic acid. The earthy phosphates are dissolved. Calcium oxalate is not dissolved and can be identified by the shape of the crystals.
- 4. To the acetic acid solution add ammonium molybdate and nitric acid, and heat to  $60^{\circ}$ C. The formation of a yellow precipitate indicates the presence of phosphoric acid.
- 5. If no precipitate forms upon the addition of ammonia in (2), the calculus is either calcium or magnesium carbonate. To a bit of the calculus add a drop of HCl. The evolution of gas (CO<sub>2</sub>) proves the presence of carbonates.
- 6. To half the ammoniacal solution add ammonium oxalate solution. The formation of a precipitate (of calcium oxalate crystals) shows the presence of calcium carbonate.
- 7. To the other half of the ammoniacal solution add sodium phosphate solution. The formation of a precipitate (of triple phosphate crystals) shows the presence of magnesium carbonate.

# CHEMICAL TESTS

Tests for Protein (serum albumin and serum globulin).—The urine must be clear. If cloudy, it must be filtered, after shaking it up with Kieselguhr if necessary.

1. Heat and acetic acid.—A thin walled test tube is 23 filled with clear urine, and the upper portion (about 1 inch in depth) is brought to gentle boiling over a free flame. A few (3-10) drops of 5% acetic acid are added drop by drop, boiling between each drop. A cloud which appears on boiling and disappears on addition of acid is due to carbonates and phosphates. A cloud which persists or increases in density after acidification is usually due to albumin.

A positive reaction may also be given by *mucin* ("nucleo-albumin"), a substance which may be secreted by the mucous membranes of the urinary passages, particularly if slightly inflamed or irritated. It may be differentiated from albumin by the fact that it is precipitated by acetic acid in the cold. It has no clinical significance.

Interference by mucin can be avoided and the test made more delicate by adding to the urine  $\frac{1}{5}$  its volume of saturated sodium chloride, and 2-5 drops of glacial acetic acid before boiling (Purdy). We regard the test thus modified as the most practicable and delicate of the ordinary procedures for routine work.

The approximate strength of the reaction may be expressed as follows: Trace: a faint cloud, clearly seen only when viewed against a dark background, best with illumination from above.  $+^1$ : a cloud which is distinct, but still transparent.  $+^2$ : a uniform opaque cloud.  $+^3$ : a dense cloud which does not immediately flocculate.  $+^4$ : immediate formation of a flocculent coagulum.

Nitric Acid Test (Heller).—In the bottom of a conical glass or wide test tube put a few cc. of concentrated nitric acid, or preferably Robert's reagent (HNO<sub>3</sub>, 100 cc.; saturated aqueous solution of MgSO<sub>4</sub>, 500 cc.). Over this layer carefully a few cc. of clear urine, by inclining the tube and allowing the urine (from a pipette) to flow slowly down the side of the tube. Examine after 3 minutes. In the presence of albumin an opaque white cloud ("ring") forms at the plane of contact. In the absence of albumin confusing rings may be formed at the surface of contact by urea nitrate crystals (avoided by diluting the urine), and by resins (soluble in alcohol). Rings above the line of contact may be formed by urates, or mucin. Colored rings without turbidity, due to oxidation of pigment or ingested drugs, should not be confusing. It is less sensitive than the preceding test, but useful when only small amounts of urine are available. Urine containing 0.0033% albumin will give a perceptible ring in exactly 2 minutes. A quantitative estimate can be made by determining the dilution of urine with 0.6% salt solution which will just give a reaction in 2 minutes, and multiplying this by 0.0033%.

A rough quantitative estimation may be made with Esbach's albuminometer when considerable albumin is present. The clear urine is added to the mark U; if not strongly acid, one or two drops of glacial acetic acid is added; and the tube is filled to the mark R with Tsuchiya's reagent (phospho-tungstic acid, 1.5 Gm.; HCl, 5 cc.; 95% alcohol, 85 cc.). The tube is mixed by gentle inversion several times, and allowed to stand just 24 hours. The depth of the precipitate is read on the graduated tube, and gives directly the protein content in grams per liter.

More accurate results (with an error usually less than 10%) may be obtained by using the specially graduated centrifuge tube of MacKay. The tube is filled to the 4 cc. mark with filtered urine (diluted to contain approximately 0.1 Gm. protein per liter), and Tsuchiya's reagent added to the 6.5 cc. mark. The tube is stoppered and inverted gently three times. After just 10 minutes it is centrifuged just 10 minutes at a speed of 1800 revolutions per minute. Calculation: Gm. protein per liter = cc. precipitate × 7.2 × dilution of urine used.

Purdy's method may be used. Put 10 cc. of urine in a graduated centrifuge tube; add 2 cc. of 50% acetic acid, then 3 cc. of 10% potassium ferrocyanide. Mix by gentle inversion, and after standing 10 minutes, centrifugalize exactly 3 minutes at 1500 revolutions per minute in a centrifuge having a radius of 634 inches with tubes extended. Albumin (in per cent by weight) = 0.21 × volume of sediment in cc.

The association of Life Insurance Medical Directors of America have adopted the following method. In a test tube graduated at 10 cc., pipette 2.5 cc. of clear urine, fill to the mark with 3% sulphosalicylic acid, and mix by inversion. After 10 minutes compare the turbidity with that of standard tubes of known protein content. Standards that are permanent for at least 8 months can be made by Kingsbury's method (1020) or can be purchased (see also p. 634).

Although it is important to have a rough estimate of the amount of albumin present, the additional information gained by precise estimations rarely repays, for practical purposes, the time spent in making them.

The use of more sensitive reagents such as trichloracetic acid for routine purposes is undesirable, as they often give a positive reaction with normal urine, and may be confusing.

Proteoses, which are protein decomposition products, may appear in the urine in conditions in which necrotic tissue or inflammatory exudates are being absorbed. They are precipitated by sulphosalicylic acid, trichloracetic acid and in part by cold nitric acid, but not by heat and acetic acid. Care must be taken to distinguish them from albumin if the former reagents are used, as their significance is entirely different.

To remove albumin as a preliminary to other tests, put a measured volume of urine in an Erlenmeyer flask, acidify slightly with dilute acetic acid if not acid to litmus, and bring to boiling. In case the albumin does not separate as a flocculent precipitate (but merely causes a diffuse turbidity), if the sp. gr. is low, add a few cc. of saturated sodium chloride solution, and again boil. If necessary add more dilute acetic acid, drop by drop, boiling after each addition. Excess of acid must be avoided. Filter until clear, and test filtrate for albumin, preferably with sulphosalicylic acid.

Significance.—A positive reaction for albumin by the usual tests is probably always pathological, but it may occur as a result of clinically insignificant or transient causes, and by no means necessarily indicates kidney disease. The significance of albuminuria depends entirely upon the condition causing it. This can only be determined by a clinical study of the patient, in conjunction with the results of urine examinations, and rarely by the latter alone.

Albumin may enter the urine through the kidney, or at any point in the urinary passages ("post-renal albuminuria"), usually as a result of local bleeding, irritation, or infection. Albumin may appear in the urine as a result of irritation or disease of the kidney (renal albuminuria) or of circulatory disturbances or other extraneous conditions ("pre-renal albuminuria"). In either case casts are usually present also, although there is no close parallelism between the number of casts and the amount of albumin present. Among the causes of pre-renal albuminuria are

congestion resulting from myocardial failure, or pressure on the renal veins by a pregnant uterus, or a tumor; severe anemia; and probably excessive muscular exercise, and cold baths.

The orthostatic albuminuria of adolescents probably belongs in this group. It is characterized by the excretion of albumin, often in large amounts, while the patient is active in the erect posture, and complete disappearance of the albumin while prone in bed. No casts or blood cells are present, and renal function is normal while prone, but in the erect posture renal epithelial cells and casts (hyaline or more often granular) may be excreted, and renal function may be impaired, as determined by the more sensitive tests (Rytand, 1937). Many cases show an exaggerated lumbar lordosis, and pressure on the renal veins has been suggested as an explanation. Others attribute it to circulatory disturbances associated with vasomotor instability and a low pulse pressure. In its slighter degrees it probably accounts for most of the "physiological" or benign albuminurias of adolescence. The prognosis is usually good, although the diagnosis must be made with caution, since posture may have a marked influence on the albumin output in true nephritis.

Renal albuminuria may be due to irritation from various drugs and poisons, notably mercurial salts, lead, phenol, turpentine, cantharides. mustard. It also occurs frequently in many acute febrile diseases, such as typhoid fever, pneumonia, scarlet fever and other streptococcal infections. without evidence of a true diffuse nephritis. It is practically constant in all forms of nephritis, including the focal nephritis of chronic sepsis, the arteriosclerotic nephropathies, amyloid kidney, and chronic pyelonephritis. as well as acute and chronic glomerulonephritis, including the "nephroses." In acute nephritis the quantity of albumin varies with the acuteness and severity of the process, is usually large, and with the presence of red blood cell casts, makes the diagnosis certain. Extremely large amounts of albumin (20 to 80 grams per liter) are seen chiefly in "nephrotic" types of nephritis, including syphilitic nephritis. In other cases there is no definite relation between the gravity of the disease and the degree of albuminuria. Inconstant traces only may be excreted in advanced stages of contracted kidney. The presence of epithelial casts and coarsely granular casts indicates active disease of the kidney with injury to the tubular epithelium, usually a nephritis (particularly if red cells are also found).

It has not been possible to make a classification of cases of nephritis which harmonizes both with the anatomical changes in the kidney and the clinical manifestations of the disease, nor to predict with any constancy from the latter, just what the autopsy findings will be. The chart shows

# CLINICAL TYPES OF RENAL DISEASE

	Urine	Specific gravity	Albumin	Sediment	Etiology	Special features
Acute glomerulo- nephritis. (Exacerbations of chronic glomer- ulonephritis.)	Diminished, Color dark, Haematuria frequent,	High, 1.025–1.030	Abundant	Abundant, Hyaline, granular, cellular, red cell casts. Red and white cells. Renal epithelium.	Follows acute infections, nearly always strepto- coccal.	Abrupt onset. Oedema fre- quently marked, especially of face; may be trivial. Hypertension, nitrogen re- terion common, May re- cover, become chronic, or terminate in uraemia.
Nephrotic stage of active glomerulonephritis. (Includes some cases of "nephrosis.")	Usually diminished, Color moderately dark.	High, I.020-I.030	Very large amount; 0.5 to 8.0 %.	Abundant as above, often fatty and waxy casts. Red cells often scanty. Doubly refractile granules often found with polarizing microscope.	Follows acute infections.	Ocdema marked, Low plasma proteins. De generative changes marked. Blood pressure usually normal. Argenia not common. Merges with (1), may terminate in (3).
Chronic glomer- ulonephritis,	Usually increased, pale, clear. Noc- turia marked.	Usually low, often fixed un- der 1.010-	Usually small amount or trace only.	Scanty. A few hyaline and granular casts and a few red cells.	Repeated acute or chronic infections. Subject to acute exacerbations.	Usually no oedema. Hyper- tension and nitrogen reten- tion usual. Albuminuric retinitis. Terminal uraemia.
Focal nephritis.	Normal in amount. Periods of haema- turia.	Normal	Slight to marked.	Red cells. Variable number of casts.	Sepsis, especially sub- acute bacterial endo- carditis.	No oedema. No hypertension or uraemia. Retinal
Artenolosclerotic nephropathy. Vascular neph- ritis.	Normal or increased. Often nocturia.	Normal or usually dimished.	Trace or small Scanty. amount. absent,	Scanty. A few hyaline casts. Red cells few or absent, rarely found.	Not known. In part heredity, obesity, ner- vous tension, wear-and- tear of life.	Hypertension and cardiac hypertension marked. Death usually from cardio-vasoralar disease. Nitrogen retention and uraemia may occur late. Atteriosclerotic retinal changes.
Chronic passive congestion.	Chronic passive Diminished. Color High congestion.	Hign	Slight or moderate.	Slight or mod- Variable. Hyaline and granular casts. May show a few red cells. Sedimentum lateritum.	Myocardial insufficiency from any cause.	No uraemia or nitrogen re- tention uniess complicated Salt excretion impaired. "Cardiac" oedema, normal plasma proteins.
Pyelitis and cyst- itis.	Normal amount, Norma: Turbid in varying degree.		Slight to marked.	Pus in varying amount. Some red cells. Bac- teria. Often a few casts (pyelonephritis).	Descending or ascending infections of urinary tract.	No oedema. Common with urethral or ureteral obstruction. May lead to hydronephrosis, pyelonephris, hypertension and uraemia.

the usual major features in typical cases of the main clinical types of nephritis. Many transitions and combinations occur.

The term "nephrosis" has been used for the sake of brevity in these discussions as a synonym for "acute degenerative non-haemorrhagic nephritis with oedema," the so-called lipoid nephrosis. The term is a misnomer as applied to these cases, since recent work has shown that there are invariably some inflammatory changes in the kidneys, and the condition can not be differentiated sharply from ordinary acute haemorrhagic nephritis with oedema.

Bence-Jones protein may be demonstrated by acidifying a clear specimen of urine with dilute acetic acid, and heating gradually in a water bath. A turbidity or precipitate which is maximal at 60°C., and which dissolves on boiling and reappears on cooling, indicates its presence. If albumin is also present it may be removed by filtering the hot urine. The Bence-Jones protein, if present, will reprecipitate on cooling. The substance appears in many (not all) cases of multiple myeloma; and rarely in leukaemia and in carcinoma or other malignant growths invading the bone marrow.

Congo Red Test for Amyloidosis.—(1) Inject intravenously one-fourth cc. per kilo of body weight (not over 18 cc.) of a 1.5% solution of Grübler's Congo red. (See Blood Volume, p. 315.) (2) Four minutes later withdraw 10 cc. of blood from the opposite arm with a clean syringe and oxalate in a graduated centrifuge tube, taking the usual precautions to prevent haemolysis. (3) One hour after the injection similarly obtain and oxalate a second 10 cc. specimen of blood. Also secure a specimen of urine. (4) Centrifugalize both the four-minute and one-hour specimens until the plasma is clear. (5) Transfer the plasma to colorimeter cups. (6) Set the cup containing the four-minute plasma at 10 mm. (regarded as the 100% standard), and match the other against it.

The per cent of dye retained =  $\frac{\text{Reading of 4 minute plasma}}{\text{Reading of 1 hour plasma}} \times \text{100}.$ 

The per cent of dye eliminated is obtained by subtracting this figure from 100%.

In normal cases less than 30% to 40% of the dye is eliminated (usually less than 20%). In "nephrosis" from 40% to 60% may be eliminated, but a considerable amount of the dye will be found in the urine, which will be deep red in color. The climination of more than 40% (usually more than 60%) of the dye from the blood without the appearance of much dye in the urine usually indicates amyloidosis. The result of the test may be misleading, however, in individual cases.

### Sugar

Reducing substances, including some fermentable carbohydrates, are present in traces (0.1% or less) in normal urine. The amount of glucose appears to be very minute. In pathological conditions larger amounts of glucose may be present; rarely also lactose, levulose, maltose and certain pentoses. All these sugars reduce alkaline copper solutions. Preservatives interfere with some of the tests. Albumin, if present, should be removed.

Benedict's Qualitative Test.—This is the best for routine use, as it is more sensitive, and gives fewer positive reactions with other reducing substances than other methods. It is not reduced by formaldehyde or chloroform.

To make the solution dissolve 173 Gm. of sodium citrate and 100 Gm. of anhydrous sodium carbonate (or 200 Gm. crystalline) in 700 cc. water (boiling water-bath), and

filter. Add slowly, with constant stirring, 17.3 Gm. CuSO<sub>4</sub> dissolved in 100 cc. water, cool and dilute to one liter. It keeps indefinitely.

Procedure: (1) Put exactly 5 cc. of the solution in a test tube, and heat to boiling (to check solution). (2) Add 0.5 cc. urine (8-10 drops only) from a pipette. (3) Mix well, and put in boiling water bath for 5 minutes or boil vigorously over a free flame for 1 to 2 minutes. (4) Remove and let cool slowly. A positive reaction is indicated by a general turbidity, due to the formation of a bulky precipitate which is greenish, yellow, or brick-red, according to the amount of sugar present; and, later, by the deposition of a yellow or red precipitate. A whitish turbidity or sediment is due to urates and is of no significance. A weak reaction may be evident only after standing. The test will detect glucose in concentration of 0.02%. A positive reaction is not specific for glucose but merely proves the presence of some reducing substance. Weak and usually somewhat atypical reactions may be obtained after the administration of many different drugs. When a reducing substance is found for the first time in the urine, it must be further identified by the fermentation test, and if this is positive, preferably also by the phenylhydrazine test.

Fermentation Test.—(1) Boil about 25 cc. of urine (free from preservative) to kill colon bacilli which may ferment lactose if that be present, and cool. (2) Rub up a piece of fresh yeast the size of a pea in the urine, and acidify faintly with turtaric acid, if necessary. (3) Put in fermentation tube, taking care that all air bubbles are expelled, and incubate a few hours. (4) If not certain that the yeast is active and free from fermentable sugar, similarly set up controls of yeast in normal urine; and yeast in normal urine to which glucose has been added in about the same percentage as that present in the specimen which is to be examined.

A positive reaction is indicated by the appearance of gas in the closed arm of the tube about equal in volume to that in the tube to which glucose was added; or if less than about 0.1% of glucose was present, merely by the failure of the fermented urine to reduce Benedict's solution.

Although this test may (rarely) give false positive reactions, in conjunction with a positive Benedict's test it practically proves the presence of glucose. Levulose, galactose and maltose (slowly) react similarly. Their differentiation from glucose is difficult and rarely of any importance, since practically they only occur with glucose in diabetes. Lactose and pentoses are not fermented. Glucose is the only reducing substance which produces a hyperglycaemia.

The phenylhydrazine test is little used for the detection of glucose, but is of value in identifying it conclusively. (1) To 10 to co. of urine add a few drops of saturated lead acetate solution and filter. (2) Acidify with acetic acid, and (3) add 1.0 Gm. phenylhydrazine hydrochloride and 2 Gm. sodium acetate, or preferably pour the urine into a tube containing five drops of basic phenylhydrazine, ten drops of glacial acetic acid, and 1 cc. of saturated sodium chloride solution (Kowarski). (4) Heat in boiling water bath 1-2 hours, or boil over a free flame, with constant shaking, until the volume is reduced by two-thirds (much bumping). (5) Replace water lost, and filter hot. (6) Allow to cool slowly in water bath without agitation. (7) If positive, under the microscope the glucosazone crystals appear as fine straight needles arranged in fans, sheaves or spherical clusters. Their appearance is practically pathognomonic. After purification by recrystallization from hot 60% alcohol they melt at about 200°C. Only a positive reaction is significant.

Levulose yields the same osazone. Similar osazones may be obtained from lactose, galactose, and maltose, but only under special favorable conditions. They can be differentiated from glucosazone crystals by their shape, maltose yielding broader, blunter crystals, and lactose, finer, curled crystals. Pentoses yield osazones melting at about 165°C., and glycuronic acid an osazone melting at 115°C.

Quantitative Estimation of Glucose.—A mixed 24 hour specimen should be obtained and its volume noted.

Benedict's quantitative solution is recommended. To prepare the solution (1) dissolve in about 650 cc. of water 100 Gm. anhydrous (or 200 Gm. crystallized) sodium carbonate, 200 Gm. of sodium (or potassium) citrate, and 125 Gm. of potassium thiocyanate. Filter if necessary. (2) In 100 cc. of water dissolve exactly 18.0 Gm. CuSO4 (crystals). (3) Pour slowly, with constant stirring, into the above solution. (4) Add 5 cc. of 4% potassium ferrocyanide solution. (5) Cool, dilute to one liter, and filter if necessary. It keeps indefinitely. 25 cc. are reduced by 0.050 grams of glucose.

Procedure.—(1) Dilute the urine 1 to 10 unless the amount of sugar is small. (2) To 25 cc. of the reagent in a 500 cc. Erlenmeyer flash, add 10 Gm. anhydrous sodium carbonate and a little powdered pumice stone, and bring to a boil. (3) From a burette add the diluted urine rapidly until a chalky white precipitate begins to form, then more slowly, a few drops at a time, until the blue color has disappeared. The flash must be kept boiling, and be constantly agitated and water lost by evaporation must be replaced.

Calculation:

$$\frac{0.050 \times 100}{\text{No. cc. of urine}} = \text{Per}$$
 glucose.

If only small amounts of urine are available, 5 cc. of reagent may be used, in a 100 cc. flask.

Calculation:

$$\frac{0.010 \times 100}{\text{No. cc. of urine}}$$
 = Per cent glucose.

Any other reducing substances present are included with glucose in these measurements.

Significance.—Recent work indicates that the fermentable reducing substances in normal urine include only insignificant traces of glucose (less than 0.01%), and consist largely of unutilizable carbohydrates from the food. The normal individual does not excrete appreciable amounts of glucose until the blood sugar rises above a definite rising threshold level. This varies with different individuals from 114 to 216 mg. % in arterial blood (Höst), but is constant in the same individual. If it rises much above this level and if the rise is maintained for an appreciable time, considerable amounts of glucose will be excreted. Once initiated, the excretion of glucose continues until the blood sugar has fallen to a level considerably below that at which excretion began—the falling threshold level. This also varies greatly and may be at or near the normal fasting blood sugar level.

Glycosuria may occur in a normal individual as a result of any of the factors which may elevate the blood sugar, enumerated on p. 661, provided the rising threshold level is exceeded. It is frequently seen as a result of powerful emotional disturbances. It occurs in some normal persons after a heavy carbohydrate meal, although in most normal individuals it is difficult or impossible to administer enough carbohydate (as starch or glucose) by mouth to produce a glycosuria.

Glycosuria may occur in a variety of pathological conditions other than diabetes. The most important ones have already been enumerated as causes of hyperglycaemia (see p. 661). The possible presence of these conditions must always be considered, but a persistant glycosuria associated with a high fasting blood sugar in an individual on an ordinary diet usually means diabetes mellitus. Absence of a glycosuria does not exclude diabetes. Apart from the effect of dietary restriction, elderly diabetics with considerable arteriosclerosis may show little or no sugar in the urine, although their fasting blood sugar is 200 mg. % or even higher (high threshold).

Lactose is often excreted in the urine of lactating women and occasionally in sucklings. Pentoses may be excreted after eating large quantities of fruit. They are also met with in a rare constitutional anomaly, "pentosuria," a condition which does not affect the health but may be mistaken for diabetes. They are non-fermentable. Galactose is rarely found except after its administration as a test of liver function. Glycuronic acid compounds are excreted in minute amounts in normal urine, but they may be increased greatly after taking certain drugs (chloral, camphor, morphine, salicylates, etc.). They may be mistaken for glucose as they will reduce Benedict's solution, but are not fermentable. Another non-fermentable reducing substance is homogentisic acid, excreted in the rare constitutional anomaly, alkaptonuria. Urine containing it blackens on standing, from oxidation.

Ketone bodies.—Acetone, diacetic acid, and  $\beta$ -hydroxybutyric acid are present in large quantities in the urine (ketonuria) in cases of diabetic acidosis. Smaller amounts of acetone and diacetic acid may be present in normal individuals or in other conditions if starved or if on diets which are very high in fat and low in carbohydrate. Ketonuria indicates incomplete oxidation of fats. The body is able to oxidize fat completely only if a certain minimum amount of glucose is simultaneously oxidized (about 1 Gm. of glucose for each 2 Gm. of fat). A deficiency may be due to lack of available glucose for oxidation (in starvation), or inability to oxidize an adequate amount of glucose when present (insufficient insulin in diabetes). The demonstration of acetone and diacetic acid in the urine in diabetes is of great practical value, in indicating the presence of acidosis. If the reaction for diacetic acid is strong,  $\beta$ -hydroxybutyric acid may safely

be assumed to be present also. However, quantitative estimations of these substances are less valuable in measuring the degree of acidosis than are other simpler procedures (e.g. the CO<sub>2</sub> combining capacity of the plasma) and are rarely worth the time required to do them.

There are other types of acidosis in which ketonuria does not occur.

Rothera's test for acetone.—To 3 cc. of urine add an excess of ammonium sulphate and shake until saturated. Add 3 drops of fresh 5% solution of sodium nitroprusside, and layer over this 1 to 2 cc. of concentrated ammonia. A positive reaction is indicated by a reddish-purple band appearing within 15 minutes. The test is delicate, and like all clinical tests for acetone reacts also with diacetic acid. A weak positive reaction has little significance.

If a positive reaction is obtained, test for diacetic acid by Gerhardt's method. (Urine which gives a negative Rothera test will never give a positive Gerhardt reaction.) The urine must be fresh, as diacetic acid is converted into acetone on standing.

Gerhardt's test for diacetic acid.—To 5 cc. of urine add 5% ferric chloride drop by drop until the precipitate which forms redissolves. In the presence of 0.05% diacetic acid a deep red color develops. As a control, repeat the test with a specimen which has been boiled to drive off the diacetic acid. (If a red color then develops it is due to other substances, which occur commonly after taking salicylates and related drugs, sodium bicarbonate, etc.) This test is not very delicate. A positive reaction indicates a significant degree of acidosis.

If there is doubt as to the outcome of these reactions, acidify 250 cc. of urine slightly with phosphoric acid and distill off 20 cc. of fluid.

Apply Rothera's test to the distillate. All diacetic acid is converted into acetone, and disturbing substances are eliminated.

Ketogenic Diet.—An artificially produced ketosis has been utilized for the treatment of epilepsy and migraine, and, recently, for urinary tract infections. In epilepsy a moderate ketosis is maintained over a long period, whereas in infections of the urinary tract an abrupt and intense ketosis should be developed over a period of about 10 days.

Ketosis may be produced by a diet which is adequate in caloric value, but in which the ratio of fat to carbohydrate is so high that the body is unable to oxidize the fat completely. The same result may be obtained by starvation, or by a diet inadequate in calories and restricted in carbohydrate. Under these conditions the body is forced to burn its own fat, and the resulting metabolic mixture is ketogenic.

The diet that is usually preferable contains approximately the required number of calories (multiply the body weight in kilos by 35) and sufficient protein to maintain the nitrogen balance (from  $\frac{2}{3}$  to 1 Gm. per kilo). The number of grams of fat and of carbohydrate required to produce ketosis may be calculated by multiplying the estimated caloric requirement by 0.1 and by from 0.01 to 0.006 respectively (Barborka). For example, the diet for an individual weighing 125 pounds (57 kilos) should contain

2000 calories and consist of from 40 to 50 Gm. of protein, 200 Gm. of fat, and from 12 to 20 Gm. of carbohydrate. In such a diet the ketogenic-antiketogenic ratio is approximately 4:1, and a ketosis will be produced within from 3 to 5 days after starting the diet. During this preliminary period the glycogen stores in the liver are depleted to provide the necessary carbohydrate. After this period the intensity of the ketosis can be adjusted from day to day by altering the amount of carbohydrate. Nausea and vomiting do not interfere, and severe reactions need not be feared, since the ketosis can be terminated abruptly by giving glucose. Since there is no disturbance in the utilization of glucose, insulin is unnecessary. Occasionally it is impossible to obtain an adequate excretion of ketones, particularly when there is any impairment in renal function.

The treatment of urinary infections by this diet depends upon the fact that the presence of the ketone bodies in the urine renders it bactericidal. Successful results have been reported in infections due to various strains of Escherichia coli, Proteus vulgaris, Aerobacter acrogenes, streptococci, staphylococci, and even Pseudomonas (Helmholz).

This bactericidal activity is due primarily to the beta-hydroxybutyric acid which must be present in a concentration of at least 0.5%. The acidity of the urine is also an important factor and should reach a pH of 5.5 or less. An increase in one of these factors may compensate to some extent for a deficiency in the other. If treatment is to be successful, therefore, the urine should be examined daily. If the urine is not sufficiently acid, ammonium chloride or ammonium mandelate may be given. Any deficiency in the concentration of beta-hydroxybutyric acid should be corrected by reducing the carbohydrate intake.

The pH of the urine may be easily tested by using chlorphenol red test papers (or strips of filter paper soaked in a 0.04% aqueous solution of the indicator). If the pH is 5.5 or less, the yellow color of the test paper remains unchanged. (If the pH is higher, the paper will turn red.) The test must be made with freshly voided urine, since the pH alters rapidly on standing.

Precise quantitative determinations of beta-hydroxybutyric acid are elaborate and time consuming, and various simplified procedures have been suggested. The following method, adapted from that of Osterberg and Helmholz, has been very satisfactory. It has been found that there is a fairly constant ratio between the different ketone bodies in the urine, and that the concentration of beta-hydroxybutyric acid can be estimated with sufficient accuracy by quantitative determinations of the acetone and diacetic acid Both of these substances give a positive Rothera test, and the amounts present can be estimated colorimetrically. Furthermore, the depth of color developed in a urine containing 0.5% of beta-hydroxybutyric acid approximates closely that produced by a 0.5% solution of acetone.

Method.—In each of two 50 cc. graduated cylinders of the same calibre (or two Nessler comparison tubes) put (1) 0.8 Gm. of ammonium sulphate; (2) 3 drops of concentrated ammonium hydroxide (Sp. Gr. 0.88); and (3) 2 drops of a freshly prepared 5% solution of sodium nitroprusside. To one tube add 1 cc. of urine, and to the other 1 cc. of a 0.5% solution of (freshly prepared) acctone. Let stand 6 minutes. Dilute to the 50 cc. mark with distilled water, mix and compare the colors immediately. If the color of the tube containing urine is paler than the standard, ketosis is inadequate. If it is darker, repeat, using urine diluted 1-2 or 1-4.

Various dye mixtures have been suggested for permanent standards, but we have found them less satisfactory than the acetone standard. Fading of the test mixture begins immediately after dilution, and the color comparison is influenced markedly by the speed used in mixing. The acetone standard fades at about the same rate as the test, and comparable readings can be obtained over a period of several minutes.

Bile Pigments.—Bilirubin and bile salts are present in the urine in any condition causing obstructive jaundice. The obstruction may be only partial, and may be due to disturbances within the liver, as well as in the ducts. Bilirubin may be demonstrable in the urine when there is no visible tingeing of the skin or sclerae.

- 1. Foam test.—On shaking the urine, the foam shows a yellow color. (Not sensitive, or specific.)
- 2. Gmelin's test (Rosenbach's modification).—(1) Shake up the urine and filter a considerable quantity through a small filter. (2) Dry paper and sediment, in air. (3) Add a drop of yellow nitric acid to paper. A positive reaction is indicated by a play of colors: first, green, then various shades of red and blue. It is better to apply the test to the (dried) precipitate obtained when bile is removed as a preliminary to Schlesinger's urobilin test, q.v. (Not sensitive, fairly reliable if positive).
- 3. Over the urine, layer a 1% alcoholic iodin solution. If positive, a green band appears. (Simple, fairly sensitive and reliable.)
- 4. Huppert-Nakayama reaction.—(t) In a centrifuge tube put 5 cc. of urine and 5 cc. of 5% BaCl<sub>2</sub> solution. (2) Centrifuge 2 minutes. (3) Decant and discard the fluid. (4) Add to the precipitate 2 cc. of ferric chloride reagent, mix, and heat just to boiling (about 10 seconds in a boiling water bath). If positive the fluid turns green or bluish green. (5) Add one drop of yellow nitric acid. If positive the color changes to violet and red.

Reagent: To 900 cc, alcohol add 4 Gm. FeCl $_{\sharp}$  and 10 cc, of concentrated HCl, and heat to boiling.

The test is sensitive (1-1,000,000), and reliable if a negative reaction is obtained with normal urine run in parallel as a control.

**Bile salts.** Hays test.—Put the cool urine in a beaker, and sprinkle finely powdered sulphur on the surface. If bile acids are present in concentration of o.ot%, they lower the surface tension, so that the sulphur sinks at once. If it floats even after gentle shaking, the test is negative. (Sensitive but not specific.)

Urobilinogen and urobilin are present in small amounts in normal urine. They are absent in cases with complete obstruction of the common bile duct. They are increased in conditions associated with increased blood destruction, or with diffuse liver injury. In freshly voided urine pigments are largely or entirely in the form of urobilinogen, which is rapidly converted into urobilin by light and acid.

Urobilinogen test.—To a small amount of fresh urine add 100 its volume of Ehrlich's reagent (2% solution of paradimethylamidobenzaldehyde in 20% HCl). Let stand to minutes in the dark. A normal urine shows a distinct but rather faint reddish tinge (much intensified by heating). When urobinogen is increased, a deep, bright red color rapidly develops. If more than a faint color is present set up a series of graded dilutions of urine (2 cc. each in small test tubes) from 1–10 to 1–200; add 0.2 cc. of reagent (do not heat); after 5 minutes inspect by looking down through the fluid against a white background. Note the highest dilution giving a distinct pink color. Normal urine is negative in dilutions higher than 1–10.

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Reagent: To 990 cc. alcohol add 4 Gm. FeCl $_{\pi}$  and 10 cc. of concentrated HCl, and heat to boiling.

The test is sensitive (1-1,000,000), and reliable if a negative reaction is obtained with normal urine run in parallel as a control.

Bile salts. Hays test.—Put the cool urine in a beaker, and sprinkle finely powdered sulphur on the surface. If bile acids are present in concentration of o.o1%, they lower the surface tension, so that the sulphur sinks at once. If it floats even after gentle shaking, the test is negative. (Sensitive but not specific.)

Urobilinogen and urobilin are present in small amounts in normal urine. They are absent in cases with complete obstruction of the common bile duct. They are increased in conditions associated with increased blood destruction, or with diffuse liver injury. In freshly voided urine pigments are largely or entirely in the form of urobilinogen, which is rapidly converted into urobilin by light and acid.

Urobilinogen test.—To a small amount of fresh urine add  $1_0$  its volume of Ehrlich's reagent (2% solution of paradimethylamidobenzaldehyde in 20% HCl). Let stand to minutes in the dark. A normal urine shows a distinct but rather faint reddish tinge (much intensified by heating). When urobinogen is increased, a deep, bright red color rapidly develops. If more than a faint color is present set up a series of graded dilutions of urine (2 cc. each in small test tubes) from 1–10 to 1–200; add 0.2 cc. of reagent (do not heat); after 5 minutes inspect by looking down through the fluid against a white background. Note the highest dilution giving a distinct pink color. Normal urine is negative in dilutions higher than 1–10.

Urobilin. Schlesinger's test.—(1) If bile is present, first remove it by adding ½ volume of 10% CaCl<sub>2</sub>, and ½ volume of saturated Na<sub>2</sub>CO<sub>3</sub> solution, and filtering. (2) To 10 cc. add a few drops of iodine solution (e.g. Lugol's), to convert all the urobilinogen and (3) an equal volume of saturated alcoholic ZnCl<sub>2</sub> solution (or 10% alcoholic zinc acetate solution), mix, and filter. Look for a greenish fluorescence, by holding tube in bright sunlight against a black background, focussing the rays on the tube with a lens. In normal urine only a faint tinge can be detected. In pathological urine there may be a deep green color. A rough quantitative test may be done by determining the highest dilution at which the greenish fluorescence can just be seen (for most workers, the endpoint is harder to determine than with urobilinogen). The method of Elman and McMaster may be used (p. 746). The solutions give a characteristic absorption band in the blue-green (see p. 699). If absence of urobilin is to be demonstrated, a considerable volume of urine must be extracted with chloroform, and the alcoholic ZnCl<sub>2</sub> solution added to the chloroform.

Indican.—(1) To 10 cc. of urine add 2 cc. of liquor lead acetate (U.S.P.) and filter. (2) To 6 cc. of filtrate add an equal volume of Obermeyer's reagent (0.1 Gm. FeCl<sub>3</sub> in 50 cc. of HCl.) (3) After 5 minutes add 2 cc. of chloroform and mix. If indican is present, the chloroform absorbs a blue color from the urine. Its presence is attributed to intestinal stasis. Its demonstration has little practical significance.

Haemoglobin. Orthotolidine test.—(1) Centrifuge 15 cc. of urine for 5 minutes at 1500 revolutions per minute. (2) Decant the supernatant fluid. (3) To the sediment add 2 drops of orthotolidine solution and 2 or 3 drops of acid peroxide solution.

A positive reaction is indicated by the appearance of a greenish blue to a deep blue color lasting one to two minutes. A positive reaction is given by a sediment containing roo red cells per cmm.

Solutions: (1) A 1% solution of orthotolidine in methyl alcohol. It keeps a year. (2) Glacial acetic acid 1 part; hydrogen peroxide 2 parts. It keeps several months. The benzidine test as outlined under Examination of Faeces may be used.

If the filtered urine is reddish tinged, it may be examined spectroscopically, for haemoglobin (see p. 699). If positive this test is specific, but it is not delicate.

Diazo Reaction.—To a little urine add an equal volume of Ehrlich's diazo reagent (see p. 764). Shake vigorously and add 2 cc. of ammonia. A positive reaction is indicated by a bright red color (not yellow or orange) in the urine and particularly in the foam. It is met with in certain severe acute infections, chiefly typhoid fever, measles, and acute tuberculosis, in which it is a distinctly unfavorable sign.

Burnam's test for formaldehyde.—To exert any antiseptic effect on the urine the concentration of formaldehyde (after administration of methenamine) must be at least 1–5000, and the urine must be acid. To test, remove albumin if present. To 10 cc. urine (body temperature) add 3 drops each of 0.5% phenylhydrazine hydrochloride and 5% sodium nitroprusside. Then allow a few drops of 20% NaOH to run down the wall of the tube and diffuse through the mixture. A positive reaction, indicating at least a concentration of 1–5000, consists of the appearance of a deep purplish-blue color, which changes rapidly to dark green, lighter green, and finally pale yellow. Smaller amounts may give a reddish color changing to a light yellow.

Demonstration of Mercury in Urine (Perlstein and Abelin).—To 500 cc. of urine add 3 cc. of egg albumin which has been brought into solution by trituration with a little of the urine. Boil and collect the precipitate on a filter. Dry the precipitate between sheets of filter paper and rub up in 10 cc. of concentrated HCl. Into the acid put  $\varepsilon$ 

small piece of clean copper foil or wire (2 inches of wire coiled up) and let stand over night. Remove the wire, using forceps, wash in water, alcohol, and ether, and dry. Put in a small clean dry test tube or sealed glass tube and heat gently until the copper has a greyish color. Remove the copper, introduce a small crystal of iodine and warm gently. If mercury is present a red precipitate of HgI2 forms on the wall of the tube. The reaction is more easily seen if a small plug of dentists' gold leaf is inserted in the tube near the copper before heating. If mercury is present, a small silvery spot of amalgam forms on the gold near the copper, and this turns red in the presence of iodine vapor. The test will detect o.1 mg. of Hg in 500 cc. of urine.

Arsenic (Gutzeit's method).—To 5 cc. of urine in a large test tube add 5 cc. of dilute HCl or  $\mathrm{H}_2\mathrm{SO}_4$ , a few drops of iodine solution and a few small pieces of zinc, all arsenic-free. Immediately put over the mouth of the tube a piece of filter paper moistened with saturated silver nitrate solution. If arsenic is present, within ten to fifteen minutes the paper will show a lemon-yellow color (silver arsenide). This will turn black if moistened with a drop of water, or more gradually as it dries in the air. Interfering substances are rare.

Lead (Klopstock and Kowarski).—Concentrate a 24 hour specimen of urine on a water bath to one lifth its volume, add an equal volume of concentrated HCl, heat to boiling, and add potassium chlorate, a little at a time, until the fluid becomes colorless. Boil off the excess chlorine and add Na<sub>2</sub>CO<sub>3</sub> until nearly neutralized. Filter and pass H<sub>2</sub>S through the filtrate. If a blackish precipitate forms, collect it on a small filter paper and wash. Transfer the paper and precipitate to a small beaker, dissolve precipitate in a little hot dilute nitric acid, dilute with water and filter. Evaporate to dryness and redissolve in a little water. Test portions for lead as follows: (1) Dilute sulphuric acid produces a white precipitate; (2) potassium chromate, a yellowish; (3) KI, a yellowish: and (4) H<sub>2</sub>S, a black precipitate. Lead may be determined quantitatively by Fairhall's method (J. Biol. Chem., Vol. 60).

# QUANTITATIVE TESTS

Total solids may be estimated roughly in grams per liter by multiplying the last two digits of the specific gravity figure by 2.6 (1.6 in small children).

Determination of Nitrogenous Constituents.—In all cases a mixed 24-hour collection should be used. If albumin is present in the urine it should be removed (see p. 716). The distilled water must be ammonia-free. The reagents are the same as those described in Chap. XXXIII for use in blood examinations. In all cases in which there is a marked discrepancy between the color of the solutions to be compared the test must be repeated, using larger or smaller amounts of urine, and the final calculations correspondingly adjusted.

Total Nitrogen (Folin-Denis).—(1) Remove albumin from urine if present (see p. 716). (2) Dilute urine: r-5 if the specific gravity is 1.001-1.016; r-10 if 1.016 to 1.030; and r-20 if 1.030 and over. (3) Pipette exactly 1 cc. of diluted urine into a large (200 × 25 mm.) Pyrex ignition tube. (4) Add 1 cc. of undiluted acid digestion mixture. (5) Boil vigorously over a micro-burner, shaking constantly, until dense white fumes appear in tube. (6) Cover tube with a watch glass, and lower flame, keeping fluid just simmering until the fluid is clear and pale bluish (at least two minutes—do not overheat). (7) Allow to cool in place 60-90 seconds, not longer. (8) Add distilled water, at first allowing only a few drops at a time to flow down side of the tube.

(9) Rinse quantitatively into a 200 cc. volumetric flask, with about 125 cc. in all. (10) In a similar flask put 20 cc. of standard N solution (containing 1.0 mg. N), 1 cc. of undiluted acid digestion mixture, and about 125 cc. of water. Add 4 drops of gum ghatti solution to each. (11) To each flask add 30 cc. Nessler's solution. (12) Fill each flask to the mark with water, mix, centrifugalize if not clear, and compare in colorimeter. For special precautions necessary see p. 651, N.P.N. in blood. Calculation: Since the standard contained just 1 mg. N, and both standard and unknown were equally diluted:  $\frac{\text{Standard}}{\text{Unknown}} \times \text{Dilution} \times \frac{\text{vol. in c.c. of total urine}}{1,000} = \text{grams of N in}$ 

24 hours. Normal is 10 to 10 grams.

Urea Nitrogen (Folin-Youngburg).—(1) Dilute urine 1-10 (if specific gravity is low, 1-5). (2) To 25 cc. of diluted urine add 4 Gm. of dry, dust-free permutit and shake gently for 5 minutes. (3) Let stand 1 minute, and decant the ammonia-free urine, or centrifuge if not clear. (4) Put 1 cc. in a 25 × 200 mm. Pyrex test tube. (5) Add urease (either 1 cc. of solution, or a square of prepared paper), and two drops of buffer phosphate solution. (6) Put in water bath at 50°C. for 5 minutes (or longer). (7) Wash quantitatively into a 200 cc. volumetric flask with about 150 cc. of water. (8) In a similar flask put 20 cc. of standard solution (containing 1 mg. N), the same urease, 2 drops of buffer phosphate solution and 150 cc. of water. (9) Add to each flask 4 drops of gum ghatti solution and (10) 20 cc. of Nessler's solution. (11) Dilute to the mark, mix, and compare in the colorimeter.

Calculation:  $\frac{\text{Standard}}{\text{Unknown}} \times \text{Dilution} \times \frac{\text{Vol. in cc. of 24-hour specimen}}{\text{r,000}} = \text{Grams}$ 

urea-nitrogen in 24-hour specimen. Normal is 10-15 grams. The figure may be converted into terms of *urea*, by multiplying the urea nitrogen figure by 2.14.

Ammonia. (Folin-Bell).—(1) In a 200 cc. volumetric flask put 2 Gm. dust-free permutit, washing down with 5 cc. of water, and (2) 2 cc. of undiluted urine, washing down with a little water. (3) Shake gently, but continuously, for 5 minutes, and rinse to bottom of flask with 25 cc. of water. (4) Let stand till clear, decant, and discard fluid. (5) Wash permutit with abundant water, and decant three times, discarding washings. (6) Add a little water. (7) In a similar flask add 10 cc. of standard N solution (containing 0.5 mg. of N). (8) To both flasks add 5 cc. of 10% sodium hydroxide, and quickly dilute to about 150 cc. (9) Add 10 cc. Nessler's solution to both flasks. (10) Dilute to mark and mix. (11) Let stand 10 minutes, and compare in colorimeter. Calculation: Since the standard contained 0.5 mg. N:

Standard/Unknown  $\times$  0.5 mg. = mg. ammonia N in 2 cc. urine used; and mg. ammonia N per 1 cc. urine =  $\frac{1}{2}$  this figure.

 $Standard/Unknown \times 0.5 \text{ mg.} \times \frac{1}{2} \times \frac{\text{Vol. in cc. of 24-hour specimen}}{r,000} = \text{grams of}$ 

ammonia N in 24 hour specimen. The normal figure is 0.5 to 1.2 Gm. per 24 hours.

Uric Acid (Benedict and Franke).—(1) Dilute a portion of the urine with water 1 to 20. (If dilute, 1 to 10. The diluted urine should contain 0.15 to 0.3 mg. uric acid in 10 cc.) (2) Put 10 cc. diluted urine in a 50 cc. volumetric flask. (3) In a similar flask put 10 cc. of standard uric acid solution. (4) To each add from a burette 5 cc. of 5% sodium cyanide solution (Poison). (5) To each add from a burette 1 cc. of uric acid reagent (Poison). (0) Mix; after 5 minutes at room temperature dilute to the mark and compare in colorimeter.

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Calculation:

$$\frac{\text{Standard}}{\text{Unknown}} \times 0.2 = \text{mg.}$$
 urea in 10 cc. diluted urine.

If the urine was diluted 1 to 20, Standard/Unknown  $\times$  0.2  $\times$  2910 = mg. uric acid per 1 cc. urine; and this figure  $\times$  24 urine vol. = grams uric acid in 24 hour urine.

Standard uric acid solution.—Dilute a little of the strong standard solution described under blood uric determinations (p. 658) 50 times with water. (For other reagents see p. 657.)

Significance.—Ordinarily estimations of uric acid in the urine are of no practical value in diagnosis. The quantity varies greatly (0.07 to 0.24 Gm. per day) with the purin content of the diet. An increase occurs in any condition in which tissue rich in nuclei is disintegrating (leukaemia, acute liver degeneration, pneumonia in resolution). In acute gout the excretion tends to be diminished before an attack and increases after it.

The Purin tolerance test yields information of some value in suspected cases of gout, if renal function is not impaired, but it is cumbersome, and adequate cooperation is rarely secured outside a hospital. The patient is kept for two days on a diet low in purins (no meats, legumes, or spinach), and total 24 hour urine collections made. On the morning of the third day, give 200 Gm. of thymus ("neck" sweetbreads); continue the purin-low diet, and urine collections for three days. Make uric acid determinations on all specimens. In a normal individual practically all the added purin is excreted in 24 hours, so that on the fourth and fifth days, the low uric acid value of the second day is regained. In gout the excretion is prolonged, and high values are found on the fourth and fifth days. The loss of a single voiding usually ruins the test.

The need for this test can be eliminated if a tophus can be found, and the sodium biurate crystals identified by the murexide test.

Murexide test.—Put a few crystals in an evaporating dish and add 3 drops of concentrated H<sub>2</sub>SO<sub>4</sub>. Evaporate to dryness by heating gently. Cool. To the reddish or yellowish residue add a drop of dilute ammonia. A positive reaction is indicated by a purplish red color which fades on warming. The crystals can not be identified by their morphology alone.

Creatinine excretion is practically constant in amount for 24 hour periods in the same individual under the same conditions. It, therefore, affords a simple method for determining whether the entire 24 hour specimen has been secured. Folin's method: (1) In a 100 cc. volumetric flask put 1 cc. of stock standard solution (containing 1 mg. creatinine). (2) In a similar flask put 1 cc. of urine. (3) To both add 20 cc. of a saturated solution of picric acid and 1.5 cc. of 10% NaOH. (4) After 10 minutes dilute to the mark with water, mix and read in the colorimeter. Calculation: Standard | Tuknown | x | 1 mg.

creatinine per cc. of urine, and  $\frac{\text{Standard}}{\text{Unknown}} \times \frac{\text{Vol. in cc. of 24 hour urine}}{\text{1000}} = \text{grams of creatinine in the 24 hour specimen.}$ 

Normal is 1 to 1.5 Gm.

Nitrogen partition.—This is determined by calculating the percentage of the total nitrogen in the urine made up by the N of the urea, ammonia, uric acid, creatinine, and the undetermined residue. The sum of the urea and ammonia N depends largely

upon the amount of protein in the food. In cases on an average diet, this figure is about 10 (to 15) Gm., or 87 (to 90) % of the total N. On a diet minimal in protein it may fall to 0.5 Gm., or 32% of the total N. (Smith 1926.) The absolute quantity of the other constituents is little affected, and their percentage figures rise. It is obvious that such determinations are worthless unless the protein content of the diet is precisely controlled, and even then no changes of clinical significance have been demonstrated except in acidosis. Here there may be a marked rise in ammonia at the expense of the urea. (Normal urea N/ammonia N ratio is about 20/1, falling on a very low protein diet to a minimum of 2/3.

Chlorides .- Volhard-Harvey method.

Solutions. (1) Standard silver nitrate solution.—Dissolve 29.06 Gm. of silver nitrate in 100 cc. of water in a liter volumetric flask. Add 250 cc. of concentrated nitric acid and 250 cc. of saturated aqueous solution of iron ammonium sulphate; dilute to the mark and mix. One cc. is equivalent to 0.01 Gm. NaCl.

(2) Standard ammonium sulphocyanate solution.—Dissolve about 6.5 Gm. of NH4SCN in just 800 cc. of water. Titrate this against solution 1, and add the amount of water calculated to be required to dilute it so that 2 cc. is just equivalent to 1 cc. of the silver nitrate standard solution.

Procedure.—(1) In a 250 cc. flask put 5 cc. of urine, 100 cc. of water and 10 cc. of silver nitrate solution. (2) If the fluid is pinkish, add drop by drop a strong solution of KMnO4 until the color is dispelled. Let stand 10 minutes or longer. (3) Add from a burette standard NH4SCN solution until the appearance of the first salmon-red tint that persists for several seconds. (If the first drop does so, add an additional 10 cc. of the silver nitrate solution, and proceed.) The red color at the end point lasts

a few seconds only. Calculation: 
$$\frac{A-\frac{1}{2}B}{5} \times 10 = mg$$
. NaCl per cc. or  $2A-B \times 10^{-5}$ 

Output/1000 = grams NaCl in 24 hour specimen. The usual normal is 10 to 15 grams, but varies very greatly with the chlorides in the food. Excretion is diminished or suppressed in most conditions in which blood chloride is reduced (pneumonia, pyloric obstruction, etc.).

Sodium chloride tolerance test.—Attempts to utilize the rate of excretion of NaCl after a test dose of 10 Gm. as a measure of renal function have not yielded results of dependable value, largely because so many extra-renal factors are involved. A normal individual who has been on an average diet will excrete the entire amount within 24 hours, but if the previous diet has been low in salt, all or most of the 10 Gm. may be retained.

Titrable acidity (Folin).—(1) In a 200 cc. flask put 25 cc. of (carefully preserved) urine. (2) Add 15 Gm. of neutral potassium oxalate and 2 drops of 1% phenolphthalein; shake well for 2 minutes. (3) Titrate to a pink color with N/10 NaOH. Calculation: Total acidity of 24 hour urine = Vol. of 24 hour urine × In normals it varies greatly with the diet, is usually 200 to 500 (Hawk and Bergheim). It is increased in acidosis (see p. 601).

True acidity is dependent on the hydrogen ion concentration, and may be determined by the method of Barnett-Chapman (p. 879). The urine should be diluted 1-5 with CO<sub>2</sub>-free distilled water, and preserved under oil until tested. The usual normal range is from pH 5.5 to 8.0. A rough estimate can be made with nitrazine test paper.

Diastase (Winslow's method).—(1) In a series of 10 small test tubes place 1 cc. of 1% NaCl. (2) Add 1 cc. of urine to the second tube, mix and transfer 1 cc. of the mixture to the third tube. Continue down the series of tubes, discarding the extra cc. in the last tube. (3) To each tube add 2 cc. of 0.1% soluble starch solution, and (4) put in water bath at 37°C. for 30 minutes. (5) Add 2-3 drops of N/20 iodine solution (or one drop of Gram's iodine solution). Determine the tube containing the smallest amount of urine which shows no blue color. The number of units of diastase = 2 ÷ volume of urine (in cc.) in this tube. The normal figure is 8-32. Excretion is diminished in nephritis, and much increased (to 100-200) in most cases of acute pancreatitis.

# Tests of Renal Function

The procedures which are of greatest practical value in demonstrating impairment of renal function are designed to show: (1) inability to concentrate the urine in a normal manner, (2) reduced capacity to excrete urea, or other test substances, and (3) the resulting accumulation in the blood of excessive amounts of nitrogenous and other waste products. Many clinical tests have been devised, of which only a few of major importance can be considered.

Disturbance of the concentrating power of the kidney is a most important manifestation of impaired function. It is the first to appear, it gives a good quantitative estimate of the degree of impairment, and can be demonstrated very simply, requiring no equipment except an accurately standardized urinometer. Patients must be under standard conditions when observations are made.

Dilution Test.—This measures the capacity of the kidney to excrete water. The day preceding the test the patient should take a normal diet without restriction of fluid, and he should drink at least a liter during the afternoon and evening. In the morning he empties the bladder and then drinks 1200 cc. of water (within 10 or 15 minutes). He may then eat breakfast, but takes no more fluid until the test is completed. Each voiding is collected separately for four hours, at least one every half hour if possible. The volume and specific gravity of each is measured.

A normal individual will excrete at least 600 cc. (often 1000 to 1200 cc.) during the four hour period, and at least one of the specimens will show a sp. gr. of 1.003 or less. The temperature of the specimens must be brought to that point at which the urinometer is calibrated. A smaller total volume of higher sp. gr. is excreted in acute nephritis. particularly in "nephrotic" cases, in chronic passive congestion of myocardial insufficiency, and in some cases of advanced chronic nephritis. In pronounced cases the total volume may be only 100 to 200 cc., and there is a tendency to fixation of the sp. gr. at about 1.018 to 1.020. The test should not be given to a patient with marked oedema.

Concentration Test.—(Unnecessary if the sp. gr. of the morning specimen is over 1.025.) As a rule this can be carried out the same day, after completion of the dilution test. The patient eats his usual meals but takes no liquids until completion of the test. Each voiding, collected every two hours if possible, up to 10 P.M., is saved separately,

and also the total night urine, preferably in two fractions. In a normal individual the sp. gr. will reach 1.030 in at least one specimen, and as soon as it does, the test is completed. If it does not, additional specimens should be collected at 10 A.M. and 12 N. the second day before fluids are resumed. A figure lower than 1.030 indicates impairment of renal function. With this there is a tendency to nocturnal polyuria and to fixation of the sp. gr. which in advanced cases may be at 1.010 or even lower.

A normal individual on an average unrestricted fluid intake excretes about 1200 to 1400 cc. of urine per day. If no fluids are taken in the evening and if the period for the collection of the night specimen is begun three hours after supper, the volume of the night urine does not usually exceed 400 cc., the sp. gr. is at least 1.016, and the ratio of the volume of the day urine to night urine is at least two to one, and is usually three to one. An increase in the volume of the night urine with a lowering of the sp. gr. and a reduction of this ratio (nocturnal polyuria) is an early and important sign of renal insufficiency.

These tests are more sensitive than any others in detecting slight degrees of renal insufficiency, particularly in essential hypertension and the early stage of arteriolosclerosis. They can be carried out on ambulant patients anywhere. Their dependability, however, depends upon the complete cooperation of the patient, particularly as to abstaining from fluids. They are not applicable to patients with urinary obstruction with residual urine or to diabetics excreting large quantities of sugar.

Phenolsulphonphthalein Test (Rowntree and Geraghty).—The test comprises the administration of the dye, determination of the interval before it appears in the urine, and of the amount excreted during definite periods. The dye is conveniently purchased already sterilized in ampoules each containing slightly more than r cc. of a solution of its mono-sodium salt, of a strength of 6 mg. per cc. (1) The patient drinks 400 to 500 cc. of water, and, (2) exactly r cc. (6 mg.) of the solution is injected twenty minutes later. Intravenous injection gives more precise results, and is to be used if the appearance time is to be determined, or if ureteral catheterization is carried out. It occasionally causes a chill, and for routine examination of ambulant patients intramuscular injection suffices. (3) The bladder is immediately emptied, and the urine saved for "backing." The succeeding specimens are usually collected by voiding. However, an accurate determination requires catheterization in patients who, for any reason, can not empty the bladder completely, or for determination of the appearance time.

The appearance time is the interval elapsing between the injection of the dye and its appearance in the urine. It is determined by allowing the urine to drip from the catheter into a receptacle containing 5 cc. of 10% NaOH, and watching for the first trace of a pink color (this urine is saved and added to the next succeeding specimen). The time interval chosen for collection of the first specimen begins at this moment, or if the patient is not catheterized, 10 minutes after the injection. (4) After an intramuscular injection the bladder is emptied after 30 minutes, again after 1 hour, and finally 2 hours after the injection. In many cases only two specimens are collected, at 1 hour and 2 hour intervals. If the volume of the first hour specimen is small another glass of water should be given then. Following an intravenous injection the first specimen should be collected after 15 minutes. The excretion of 25% or more of the dye in this specimen indicates a normal output, and for routine purposes further collections can be dispensed with.

The percentage of the dye excreted is determined by comparing the color of the urine, alkalinized and suitably diluted, with that of a standard solution. We prefer to follow

the suggestion of Cabot, and use standards in test tubes rather than in an ordinary colorimeter because the diluted urine is more or less off-color and it is impossible to make a satisfactory match without backing (if this is neglected the readings are too low).

Prepare a 100% standard by diluting exactly 1 cc. of the dye solution to 1 liter with distilled water to which 5 cc. of 10% NaOH has been added. Select 12 or 15 test tubes of identical internal diameter (check this by adding just 10 cc. of water to each tube and take those in which the fluid rises to exactly the same height; dry, and reserve them for this purpose only). Put 10 such tubes in a rack, and add to each increasing quantities of the 100% dye solution, from 1.5 cc. to 6.0 cc. (each tube receiving 0.5 cc. more than the preceding tube). Bring total volume of each to 10 cc. with alkalinized distilled water. If these tubes are tightly stoppered and kept in the dark they will keep for several weeks, but must be renewed frequently, as the color gradually fades. Label them 15% to 60% respectively.

Each specimen of urine is rinsed into a 1000 cc. cylinder, noting its volume. . Add z cc. of 10% NaOH and dilute to a definite volume, usually 500 cc. or 1000 cc., preferably so that the color approximates that of the standard tubes in the middle of the series. Mix, filter if not clear, and pour some into another of the test tubes. In another of the tubes put some of the urine obtained before injection, diluted with water to the same extent as the specimen to be tested. Put this tube behind the standard tube, for backing, and a tube of water behind the urine tube. Read the percentage figure on the standard tube which matches the color of the urine; multiply this figure by the volume (in cc.) to which the urine was diluted, and divide by 1000. The volume of the specimen does not enter into the calculations, and within wide limits the rate of excretion is independent of the volume. If the volume is quite small, the test loses in precision, If an ordinary colorimeter is used, a fresh standard must be prepared for each test, and in diluting it sufficient normal urine must be added to make its color correspond exactly to that of the unknown before alkalinization. Differences in rate of absorption, completeness with which the bladder is emptied, and other uncontrollable factors introduce unavoidable variations of at least  $\pm 5\%$  to 10% in the result, and attempts to secure great precision in the readings are pointless. A bizarre result necessitates a repetition of the test.

The normal figures, after intravenous injection, are: Appearance time: 4-6 minutes. After 1/4 hour: Minimum 25%; maximum 50%; average 35%.

After ½ hour: Minimum 40%; maximum 60%; average 55%. After 1 hour: Minimum 50%; maximum 75%; average 65%. After 2 hours: Minimum 55%; maximum 85%; average 70%. After intramuscular injection: appearance time: 10 minutes. After ½ hour: Minimum 25%; maximum 40%; average 30%. After 1 hour: Minimum 40%; maximum 60%; average 45%. After 2 hours: Minimum 55%; maximum 80%; average 65%.

Peters and Van Slyke regard a total output in 2 hours of 55% or over as within normal limits. A delayed excretion, with an increased proportion of the dye in the second hour specimen, is abnormal even though the total output is normal. Unusually high figures with diuresis have been noted in hyperthyroidism, and in mild renal disease, and have been attributed by some to renal irritation.

The great advantages of the test are its simplicity and harmlessness. Among the disadvantages is the fact that the dye is a foreign substance, and the capacity of the kidney to excrete it need not parallel precisely its capacity to excrete urea, although within limits it does roughly do so. It is not a sensitive indicator of lesser degrees of impaired function. Van Slyke et. al. have shown that in nephritis there is no notable reduction in excretion until 50% of the renal function is lost, as determined by the urea clearance test. As function is further damaged, there is a progressive fall in excretion, reaching zero in the most advanced cases. They also point out that with improvement in acute nephritis, a rise in dye excretion antedates an improvement in the urea clearance, and furnishes an early basis for a (relatively) favorable prognosis. The excretion may be reduced in other conditions which lower kidney function, such as chronic passive congestion and prostatic obstruction.

Urea clearance test—(Möller, McIntosh and Van Slyke.)

Procedure.—(1) The patient eats his usual breakfast at 8 A.M. without tea or coffee, but water as desired. (2) At 9 A.M. empty bladder completely and discard urine. Give 100 cc. water. (3) At 10 A.M. empty bladder completely, saving the specimen, and give 100 cc. water. (4) At once secure blood for urea estimation. (5) At 11 A.M. again empty bladder completely and save the specimen. The intervals need not be exactly 1 hour provided their length is precisely known. The patient should rest quietly during the test, and avoid much exertion before the test. (6) Measure volume of each specimen and calculate output per minute in cc. (7) Determine urea concentration in each specimen. (8) Determine urea concentration in blood.

Calculation: If the volume of urine excreted is more than 2 cc. per minute calculate the maximal clearance  $(C_m)$  by the following formula.

U = urea concentration in urine.

B = urea concentration in blood.

V = volume of urine excreted per minute.

 $C_m = U \times V/B$ . The average normal figure is 75, and the percentage of the normal =  $r.33 \times U \times V/B$ .

If the rate of excretion is less than 2 cc. per minute, the standard clearance (C<sub>8</sub>) is calculated according to the formula  $C_8 = U \times \sqrt{V}/B$ . The average normal (with a urine volume of 1 cc. per minute) is 54, and the percentage of normal  $= \frac{1.85 \times U \times \sqrt{V}}{B}$ .

The chief source of gross error is incomplete emptying of the bladder. By making separate estimations on the two specimens a partial check on this is obtained, and if the results correspond fairly well, the average may be taken.

For children weighing less than 100 pounds the formulas must be corrected by substituting for V in each case the value of  $\frac{V \times 1.73}{\text{Surface area in Sq. M.}}$  (from the standard DuBois height-weight tables for surface area). (Table, page 783.)

Table Giving the Value of  $\sqrt{V}$  for Varying Values of V (CC. per Minute)

V, cc.	√Ÿ	V, cc.	$\sqrt{\overline{V}}$	V, cc.	$\sqrt{\overline{V}}$
O. 2	0.45	0.9	0.95	r.6	1.27
0.3	0.55	1.0	1.0	1.7	1.30
0.4	0.63	I.I	1.05	1 8	1.34
0.5	0.71	1.2	1.10	1.9	1.38
0.6	0.78	1.3	1.14	2.0	1.42
0.7	0.84	1.4	1.18	2.1	1.45
0.8	0.89	1.5	1.23	2.2	1.48

Explanation: By urea clearance is meant the volume of blood which is entirely cleared of urea in 1 minute, or more precisely, the volume which would be so cleared if all the urea excreted in a minute were abstracted solely from one portion of blood. (As a matter of fact the blood flowing through the kidney is not entirely cleared; normally, even with high urine volume. it is probably not more than half cleared, but this does not affect the reasoning on which the test is based.) If the rate of urine secretion and other conditions are constant, the amount of urea excreted per minute varies directly with the concentration of urea in the blood. If it were possible suddenly to destroy half the functional capacity of the kidneys without altering other factors, the rate of urea excretion would fall 50%, and the previous rate of excretion would not be regained until sufficient retention had occurred to raise the blood urea to twice its initial figure. Then equilibrium would again be restored. However, other factors than the height of the blood urea do influence urea excretion, the most important being the rate of urine excretion (volume in cc. per minute). If the rate of urine excretion is slow, the total urea excreted per minute is relatively small, (even though its concentration in the urine is normal). As the volume of urine per minute rises, for a time the rate of urea excretion also rises (although not directly in proportion to the volume, but approximately in proportion to the square root of the volume). However, eventually a rate of urine excretion is reached, "the augmentation limit," about 2 cc. per minute, at which the maximum possible rate of urea excretion is obtained. Further increases in diuresis have no effect on urea output. This fact explains the necessity for the two formulas, and is the basis of the major inaccuracy of the old coefficient of Ambard. maximum clearance (75 cc. for normals) is constant, as long as the volume per minute exceeds 2 cc. The standard clearance is a variable, varies with the volume per minute, and the figure 54 applies (for normals) only to an excretion rate of 1 cc. per minute.

There are other factors, usually of less significance, such as the rate of blood flow through the kidney, which also affect urea excretion, and there are individual variations in normal persons which may cause variations in the clearance of at least 20% above or below the average figures. Reductions of less than 25% are not necessarily pathological.

Clinical significance.—Within the limitations mentioned the test gives a valuable estimate of renal function. No claims are made for exact mathematical precision, and slight fluctuations are meaningless. It is questionable whether it will detect minor degrees of impairment as early as concentration tests. It does detect them much earlier than the phthalein test. It is not reduced in the earlier stages of essential hypertension and arteriolar renal disease, and the fall in clearance as the disease progresses may be very gradual. Significant nitrogen retention does not appear until the clearance is below 40%, and is not marked until it is below 20%. Uraemic symptoms appear as the clearance falls from 10% to 5%. The clearance figure rises as a patient recovers from an acute attack. The urea clearance figures are regarded by many as giving the most precise and dependable measure of renal function in nephritis, obtainable with any single method.

The ratio of the urea concentration in the urine to that in the blood may be substituted for calculation of the urea clearance in patients from whom it is impossible to secure complete emptying of the bladder, provided the fluid intake is so adjusted as to give a rate of urine excretion of about 1 cc. per minute. In this case the value of V becomes unity, and the simplified formula  $C_s = U/B$  applies. Even moderate variations from 1 cc. alter the value of  $C_s$  (normal 54) but slightly. The results are more variable (when repeated in the same individual), and therefore less satisfactory as a routine procedure, than the urea clearance figures, but usually adequate, especially if several observations are made. It is equally sensitive in detecting slight impairment of function. It is not valid with larger urine volumes.

Urea concentration test.—This has been suggested as a simple procedure which avoids determining the urea in the blood. The patient is allowed no fluid after 10 P.M. In the morning the bladder is emptied, the urine being discarded, and he is given 15 Gm. of urea dissolved in 100 cc. of water, (flavored to disguise the nauseating taste). The total urine is collected for three one-hour periods, and measured. If the volume of the first specimen exceeds 120 cc. and that of the later ones, 100 cc., the test is useless. Otherwise the concentration of urea is determined in each. Normally it should be between 2.5% and 3% in at least one specimen. A concentration below 2% definitely indicates renal insufficiency. The converse is not true; a normal concentration figure can be reached with damaged kidneys if the blood urea is high enough. It is regarded as a comparatively gross test, although it usually shows renal injury well before nitrogen retention occurs.

An increase in the content of the non-protein nitrogeneous constituents in the blood is also a valuable indication of renal insufficiency. Of these the uric acid is often the first to rise, and was regarded by Myers as the first evidence of damaged kidney function. However, the rise is inconstant and relatively slight; it does not parallel the degree of renal insufficiency; may be normal when the urea is high; and may be equally increased in a variety of other conditions (see p. 658).

The urea nitrogen and total non-protein nitrogen are increased in the more advanced stages of renal insufficiency, in extreme cases to ten times the normal value. They are of no value in early diagnosis; and even in marked renal insufficiency may, for a long time, be kept within normal limits by a low protein-high carbohydrate diet (see p. 654). Creatinine is usually stated to be the last to rise, although Peters and Van Slyke and others have shown that in nephritis it rises about in parallel with urea. However, a significant rise is scarcely ever seen except in advanced stages of renal insufficiency, and a figure over 5 mg. % like a N.P.N. over 100, or a urea nitrogen over 80, usually indicates impending uraemia. However, in acute nephritis and in mercuric chloride poisoning the ultimate prognosis is not necessarily unfavorable even when the N.P.N. and creatinine are very high. In renal insufficiency with high blood urea due to urethral obstruction, without diffuse nephritis, Patch and Rabinowitch (1928) found no corresponding rise in creatinine, and suggest creatinine determinations as a means of determining the existence of nephritis in such cases.

In judging the functional capacity of the kidney, it is always advisable to use more than one of these procedures. The results are of great value in diagnosis, prognosis and treatment when interpreted in connection with the whole clinical picture. They are of value in surgical practice, notably in patients with prostatic hypertrophy or other conditions causing obstruction, by demonstrating the need for preoperative treatment, and determining its effectiveness. It is often possible, without unnecessary delay, to postpone operation until renal function has improved, and the operative risk is correspondingly reduced. Cases with a urea nitrogen over 30 mg. or a phthalein excretion under 40% in 2 hours (if collected by catheter) are unfavorable surgical risks.

These tests show quite accurately the function of the kidney at the moment, but they do not, by themselves, accurately portray the anatomical condition of the kidney, or furnish a basis for a dependable prognosis. A marked reduction in function may occur with circulatory failure or with severe anaemia, yet normal function may be restored if the latter conditions can be remedied. An extreme reduction in function may be met with in acute nephritis, with subsequent complete recovery. On the

other hand, a practically normal function may be found in the early stages of a chronic nephritis which progresses to a fatal termination within a year. The tests are relatively little affected by even extensive focal lesions of the kidney. Many cases of urethral obstruction with marked impairment speedily recover almost normal function under treatment, if there is no complicating diffuse nephritis. Successive tests which show a definite upward or downward trend are of great prognostic value. In acute nephritis Peters and Van Slyke point out that the degree to which the urea clearance is lowered at the onset is relatively unimportant, but the prognosis is very unfavorable if normal figures are not regained within four months. In chronic nephritis with a urea clearance under 20%, a majority of the cases die within a year, and practically all within two years. However, patients with polycystic kidneys may live a long time with a renal function reduced nearly to zero.

# BACTERIOLOGICAL EXAMINATION OF THE URINE

Collection of Specimens.—Although a catheterized specimen of urine is preferable for culture, practically it is rarely necessary in men and can often be dispensed with in women provided adequate precautions are observed in collecting the specimen and provided cultures are made immediately on solid media.

The bladder should be full at the time of the examination. The area about the urethral orifice should be thoroughly washed with soap and water, and then with 70% alcohol (men) or with sterile salt solution or boric acid. The anterior urethra may be flushed with sterile water. The first three-quarters of the urine passed is discarded and the last portion caught directly in a sterile wide-mouth flask or bottle as it flows in an unimpeded stream from the orifice without touching the skin. The urine first passed serves adequately to flush out the urethra. The use of antiseptics is not desirable.

In the absence of infection plates made in this way are usually sterile or show at most one or two contaminating colonies. If there is an infection of the urinary tract, the infecting organism is usually present in such predominant numbers that it is easily distinguished from a possible contaminating colony. With some experience contaminating organisms from the air can easily be recognized as such. A confirmatory culture should be made from a catheterized specimen if there are a few colonies present which may be pathogenic, or in any case if cultures are to be made in fluid media.

Before making the culture it is advisable to examine a stained film from the centrifugalized sediment, since this may give a hint as to the type of organism present and as to the quantity of material to be spread on the plates. After centrifugalizing, the urine is decanted, and the tube drained by inverting it on filter paper for a few minutes. A little sediment is removed with a capillary pipette, smeared, fixed by heat and stained by Gram. If the urine does not contain albumin, to secure fixation of the film add a

trace of egg white or *sterile* blood serum to the sediment, or a few drops to the urine before centrifugalization. The presence of phagocyted bacteria in pus cells is of diagnostic significance.

Acid fast organisms.—For the demonstration of tubercle bacilli, smears may be prepared by the method described, but centrifugalization must be carried out at high speed for half an hour to one hour. If the sediment is abundant it should be digested with antiformin. To avoid contamination with smegma bacilli, the same aseptic precautions required for cultures are indispensible. Smegma bacilli are not digested by antiformin. Special methods of staining to ensure decolorization of smegma bacilli are described in Chap. IV, but are not entirely dependable. If there is doubt, cultures should be made on suitable media and a guinea pig should be inoculated. Tubercle bacilli may be quite sparse in such smears and protracted search is often required to find them. When found they are apt to be in clumps. Frequently, they are morphologically less typical than in smears from sputum.

A pyuria without bacteria easily recognized microscopically or culturally should suggest tuberculosis.

Ellerman and Erlandsen have devised a reliable method for the detection of tubercle bacilli in urine which has been used extensively.

- a. Having washed genitalia and irrigated urethra, draw urine by catheter; allow to settle several hours.
  - b. Decant. Take 20 cc. of sediment; centrifuge and decant.
- c. Sediment is mixed with four times its bulk of 0.25 % solution of sodium carbonate and 0.5 Gm. digestive pancreatin; incubate 24 hours.
  - d. Upper layer of fluid is decanted; remainder is centrifuged.
- e. Decant fluid. To sediment remaining, add four times its volume of 0.25% NaOH, and stir until sediment is dissolved.
- f. Heat to boiling over a water bath for several minutes. Let cool. Centrifuge. Make smears from sediment and stain, preferably by Spengler's method.

Cultures are best made by pouring a little urine on the surface of one or two blood agar plates, and spreading with a bent glass rod. If the object of the culture is merely to demonstrate staphylococci or colon bacilli, plain agar is equally good. If typhoid or paratyphoid is suspected, Endo's agar or litmus lactose agar helps to differentiate them from colon bacilli. To cultivate *Brucella abortus* an increased CO<sub>2</sub> tension is necessary.

The amount of urine to be used depends upon the estimated number of organisms present. A satisfactory procedure in the average case is to put one drop on the first plate and spread; and five drops on the second plate and spread with the same rod, without intervening sterilization. If organisms are abundant, the urine should be diluted 10 or 100 times before plating. If a sterile culture is anticipated, 1 cc. should be poured on one of the plates. To secure a growth of gonococcus the culture should be made immediately after obtaining the material from the patient.

The organisms most often met with in infections of the urinary tract are the colon bacillus, staphylococcus, streptococcus, gonococcus, proteus, typhoid and paratyphoid bacilli, Brucella, and the tubercle bacillus. In most cases infection is probably derived from the blood stream. The infection may be ascending from the lower urinary passages to the kidney, particularly in cases starting after instrumention or associated with obstruction to the urinary passages, as by an hypertrophied prostate or urethral stricture ("surgical kidney").

Pyelitis, often without characteristic symptoms, is a common cause of chronic recurring fever, particularly in pregnant women and in girls. A persistent cystitis is practically always associated with a pyelitis.

Cystitis from a colon infection gives an acid urine; that caused by proteus, an alkaline urine.

Bacterial infections of the urinary tract are associated with more or less pyuria. In general infections, particularly with staphylococci, streptococci, typhoid and paratyphoid bacilli, the organisms may appear in the urine in large numbers without necessarily causing any notable lesion in the kidney. They may disappear from the urine if the patient recovers from the infection.

No significance can be attached to the presence of bacteria, even in large numbers, in unsterile specimens of urine which have stood in the laboratory for any appreciable time. Yeasts and moulds frequently contaminate urine, especially diabetic urine, after it has been passed.

Parasites.—Trichomonas vaginalis is a common cause of acute vaginitis in women and frequently gets into the urine in such cases.

In chylous urine filarial larvae may be found, most readily in the centrifugalized sediment.

The vinegar eel may be found in the urine of women who have used vaginal douches of vinegar.

Enterobius from the vagina may be found in the urine.

The larval dibothriocephalid, *Sparganum mansoni*, has been reported three times in the urine (urethra).

The eggs of *Schistosoma haematobium* (with a terminal spine) and rarely those of *S. mansoni* (with a lateral spine) may be found in the urine.

The eggs of Macracanthorhynchus hirudinaccus may be recognized in the urinary sediment by their pitted appearance.

Various mites have been found in urinary sediment but are accidental contaminations.

### CHAPTER XXXVI

## **EXAMINATION OF THE FAECES**

THE macroscopic, microscopic, and chemical examination of the faeces gives important information in many diseases affecting the gastrointestinal tract. In the tropics this is a procedure of major importance because of the great frequency and gravity of intestinal parasitism.

The shape, size, and consistence should be noted. The normal stool is formed but soft enough to be plastic. Soft or fluid stools indicate hypermotility and usually show incomplete digestion of food. Hard stools of large calibre indicate stasis, and usually atony of the colon. Small scybalous masses and stools of small calibre indicate spasm of the colon. Pencil or ribbon shaped stools suggest rectal stricture (cancer, syphilis) but may be due to spasm. In cholera and, occasionally, other violent diarrhoeas the stool is typically watery with little or no faecal material, but rather opaque from desquamated intestinal epithelium (rice-water stools). In amoebic dysentery there are small, frequent, mucous or muco-sanguinolent stools, often greyish-brown and homogeneous, whereas in bacillary dysentery the muco-purulent stool is streaked or flecked with blood or blood-tinged mucus. Tormina and tenesmus accompany both types of dysentery.

The *motility* of the gastrointestinal tract may be checked most simply by administering a capsule containing 10 grains of charcoal or carmine. Normally this appears within 24 to 36 hours and is entirely eliminated after 48 to 72 hours.

The normal brown color of the stool is due to urobilin (hydrobilirubin) and urobilinogen, derived from the bile pigments. Bilirubin is not normally present below the ascending colon. In the presence of diarrhoea, especially in children, the faeces may be green (biliverdin) or golden yellow (bilirubin). A pale, greasy, putty-like ("acholic") stool is characteristic of catarrhal jaundice and of mechanical obstruction of the common duct. A similar appearance, however, may be met with in gross disturbances of fat digestion (sprue, idiopathic steatorrhoea, grave pancreatic disease). The stools are usually bulky, and soft or fluid, and fat may separate out in macroscopic drops or even in large masses ("buttery stools").

A black, tarry, viscid stool indicates bleeding from the upper gastrointestinal tract. A diffuse, red-brown or bright red color indicates bleeding from the lower bowel. Streaks of fresh blood on the surface of a formed stool are usually due to hemorrhoids or rectal ulcerations (cancer, syphilis, ulcerative colitis).

The color varies markedly with the diet, and medication may give bizarre appearances.

Mucus is frequently present in large, translucent strands or sheets or jelly-like masses in cases of "spastic colitis" ("mucous colitis"), as a result of disturbances of innervation. This is characterized microscopically by the presence of numerous epithelial cells and often eosinophiles, without pus cells. These masses have often

been mistaken for tape worms by inexperienced observers. In bacillary dysentery, ileocolitis, and intusseception the stools may consist largely of mucus in greyish, opaque masses containing many pus cells and often blood.

Frothy stools indicate faulty digestion and fermentation of carbohydrates, and are especially characteristic of sprue.

In selected cases macroscopic examination should be made for gall stones, or for tape worm segments or other intestinal parasites after a vermifuge. The entire faeces (for several days if necessary) are ground up in water to from a homogeneous thin suspension, and this is strained through a sieve. The residue, suspended in water in a thin layer in a flat dish, is inspected over a dark background with the naked eye or with a hand lens. Suspected objects are removed for study. Small round worms must be differentiated carefully from vegetable fibres and from insect larvae which may occur as a contamination. For identification see section on Animal Parasites.

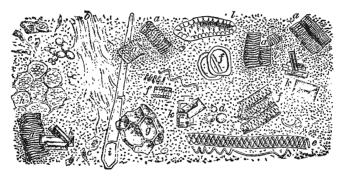


FIG. 182.—Microscopical constituents of faeces. (v. Jaksch.) a, Muscle fibers; b, connective tissue; c, epithelium; d, leukocytes; c, spiral cells; f, g, h, i, various vegetable cells; k, "triple phosphate" crystals; l. woody vegetable cells; the whole interspersed with innumerable microorganisms of various kinds.

Gall stones can usually be recognized by their facetted surface and laminated structure. Faecal concretions ('énteroliths''), composed of masses of food residue encrusted with salts, may be confused with them. Chemical tests for cholesterin and bile pigments should be carried out. (1) Crush a portion of the stone and extract with a little ether. Pour off the ether, add one half volume of alcohol, and allow to evaporate slowly. Cholesterin if present will separate as flat, rhombic plates. A drop of strong sulphuric acid causes the appearance of a red color; a drop of Lugol's solution, a play of colors. To test for bile pigments extract the residue with cold dilute KOH, and apply Gmelin's test (p. 725).

Schmidt's test diet should be administered for several days in selected cases in which it is desirable to study the completeness of digestion of the various types of food stuffs. Results are unsatisfactory if the diet is uncontrolled. The presence of oil or barium sulphate makes a specimen practically useless for microscopical examination. A charcoal capsule is administered at the start to indicate when the study of the faeces should begin.

Diet.—Breakfast, 7 A.M., bowl of oatmeal gruel (40 Gm. oatmeal, 10 Gm. butter. 200 cc. milk, 300 cc. water). Also one very soft-boiled egg (1 minute) and 50 Gm. zwieback. In the forenoon, 500 cc. of milk.

For dinner, 2 o'clock, chopped beef broiled very rare (125 grams with 20 grams butter poured over it). Also a potato purée (200 grams mashed potato, 50 grams milk. 10 grams butter). Also ½ liter of milk and 50 grams zwieback.

For supper, 7 o'clock, the same articles as for breakfast.

This detailed diet may be varied to suit circumstances as regards interchanging meals. Furthermore, the milk may be taken in the form of tea or cocoa or cooked with the other food. Even a small amount of wine may be permitted. The diet

taken, however, should absolutely conform to the following requirements: (1) The taking of 1/4 pound chopped beef, a portion of which should be half raw; (2) the milk taken should amount to about a quart: (3) about 4 ounces of bread or toast and from 4 to 8 ounces of potato purée should be eaten daily.

The detailed diet contains about 110 grams protein, 105 grams fat and 200 grams carbohydrates and has a fuel value of 2247 calories.

The stool is best collected in quart fruit jars and examined as soon after evacuation as possible. The wooden spatula-like tongue depressors are well adapted to handling the specimen.

The observer must be familiar with diet before attempting to interpret patho- Chemistry.") logical findings.

Fig. 183 .- A, neutral fat; B, fatty acid liberated by acetic acid; C, soaps; D, fatty the faeces of normal individuals on this acid crystals. (From Hawk's "Physiological

Macroscopic Examination.—Grind up a faecal mass 12 to 1 inch in diameter in a mortar, gradually adding water until it has the consistency of a broth. About 0.5 cc. of this emulsion should now be squeezed out between two slides and studied against a dark surface, and also when held up to the light. The normal stool gives a rather uniform, brownish, homogeneous layer. Connective-tissue remnants (indicative of gastric derangement) show as whitish fibres which swell and become translucent if 30% acetic acid is added. Undigested muscle tissue remnants appear as reddish brown splotches, fat particles as whitish-yellow clumps. Potato remnants appear like sago grains and mash out easily like mucus. Mucus is best noted in the faecal mass before making the emulsion.

Microscopic Examination.—(1) Muscle fibres normally appear as homogeneous, vellowish particles. Rectangular ends, distinct cross-striation, and particularly intact nuclei indicate inadequate digestion (azotorrhoea). (2) Rub up a bit of faeces with 50% alcohol, add a drop of a saturated solution of Sudan III in equal parts of 70% alcohol and acetone, and apply a cover slip. Neutral fat appears as highly refractile droplets or yellowish flakes which stain orange to bright orange-red. Normally no appreciable amount is present. Fatty acids appear as flakes which stain faintly, or fine needles which

tend to aggregate in clusters, do not stain, and melt on gently warming the preparation. Soaps appear as yellowish flakes, rounded or gnarled bodies everted like the pinna of the ear, or coarse crystals which do not melt on warming. They do not stain with Sudan III.

In normal cases the only fat elements recognizable microscopically are yellow calcium or colorless soaps. With an increased quantity of fat (steatorrhoea) droplets of neutral fat appear, together with needles and splinters of fatty acids and soaps, and often an increase in the number of soap masses. In sprue from 25% to 30% of the fat ingested appears in the stool, whereas the stool of pellagra, a disease possibly confused with sprue, shows only about 5%, the normal figure.

- (4) Vegetable cells and fibres, often of bizarre appearance, are always present in faeces of patients on a mixed diet. Familiarity with them is necessary to avoid confusing them with ova and parasites.
- (5) Pus cells indicate an inflammatory or ulcerative lesion of the intestine, but are sparse in amoebic dysentery. They should be looked for in flecks of mucus and not in the body of the stool. Except with active diarrhoea, well preserved pus or blood cells indicate a lesion in the lower colon. Many large phagocytic cells, which may superficially resemble amoebae, together with pus cells, are highly suggestive of bacillary dysentery.
- (6) Crystals.—Charcot-Leyden crystals, which are practically never found in the faeces in bacillary dysentery, are not infrequently present in amoebic dysentery. They occur also in helminthiasis and in allergic conditions, including some cases of mucous colitis. Haematoidin crystals may occur after haemorrhage. Triple phosphate and calcium oxalate crystals are often found.
- (7) Intestinal protozoa.—To demonstrate motile free-living amoebae, the faeces must be collected uncontaminated by urine or antiseptic solution and kept at body temperature until the examination is completed. It should be examined promptly. A warm stage may be used, preferably thermostatically controlled. If a fluid diarrhoeal stool is available, a fleck of mucus should be fished out and examined (16 mm. objective). Portions of a formed stool may be emulsified in salt solution and examined, but motile forms are not often found unless flecks of mucus can be secured. It is better to pass a rectal tube and examine mucus caught in the eye of the tube. If this is not successful give a saline purge, and examine the first fluid stool, securing some mucus if possible. Positive recognition requires observation of definite and typical motility. It is helpful to add a loop of 0.5% neutral red in salt solution, which vitally stains the amoebae.

Cysts are looked for in formed faeces. The material need not be fresh or warm. A moderately thin suspension through which newsprint can just be read is made in solt solution and examined with the low power. Cysts are easily located as sharply outlined, highly refractile, round structures with a diameter from one to two times that of a red cell, and can be identified with the high dry objective. They may be more easily recognized by the average observer if the drop of faecal suspension is mixed with a drop of Lugol's solution. The cysts are tinged yellowish or brownish, and the nuclei are more distinct. Some prefer to use Wenyon's iodine-eosin solution which stains the background and most other structures pink. See section on Amoebiasis.

Iodine-eosin solution.—2 parts of a saturated solution of eosin in physiological salt solution; I part of a saturated solution of iodine in physiological salt solution containing 5% potassium iodide; and 2 parts of physiological salt solution.

The flagellates (Giardia, Trichomonas, Chilomastix) and Balantidium coli can be found in ordinary fresh preparations.

The ova of intestinal helminths, if numerous, can be found in ordinary fresh preparations (using the low power objective). If they are sparse, concentration procedures are necessary. De Rivas' method (1928) is one of the simplest. (1) Thoroughly suspend I Gm. of faeces in 5 cc. of 5% acetic acid in a test tube, stopper, and shake. (2) Let stand 30 seconds, until the heavy particles settle. (3) Decant the supernatant fluid, filtering it through two layers of gauze, into a centrifuge tube. (4) Add an equal volume of ether, shake gently, and centrifuge for a few minutes. The upper ether layer may be used to test for occult blood. The bacteria and most of the detritus are suspended in, or floating on the surface of the dilute acid solution. Ova, cysts, and other organized structures are in the scanty sediment in the bottom of the tube. This is removed with a capillary pipette and examined.

More effective procedures especially applicable to hookworm are described in that section. Concentration methods should supplement and not replace the usual methods of examination.

(9) Bacteria, chiefly dead, are present in enormous numbers. Except as a means of demonstrating specific pathogenic organisms, but little useful information is to be obtained from their study.

Microscopic examination.—Suspend a bit of faeces in 70% alcohol in the concavity of a hollow ground slide. Allow the heavy particles to sediment for a few minutes, and make films of the suspension. Fix by heat or in methyl alcohol to eliminate fat, and stain by Gram's method, and for tubercle bacilli if indicated. In the normal infant Gram-positive organisms predominate (lactic acid bacilli), but in the adult most of the bacteria are Gram-negative. Gram-positive bacilli include: (1) Lactic acid bacilli, slender rods showing Gram-negative areas. They may be greatly increased in number by feeding "acidophilous milk." (2) Boas-Oppler bacilli may be very numerous in cancer of the stomach. (3) Gas bacilli: a marked increase has been held to indicate abnormal intestinal putrefaction. (4) Numerous Gram-positive cocci suggest an ulcerative lesion.

To demonstrate tubercle bacilli, select particles of mucus or purulent material if possible. They can often be found in patients with pulmonary tuberculosis who swallow the sputum, without any intestinal lesion.

Cultures to demonstrate organisms of the typhoid-dysentery group should be made on plates of special media such as Endo's, or one of the brilliant green, eosin-methylene blue, or bismuth agar media. (See section on media.) The surface of the medium must be dry. The faeces should be freshly collected in a sterile container. Rub up a small amount (the size of a pea) in a tube of sterile broth or salt solution. Transfer several loops to a plate near one margin. With a sterile platinum wire or fine glass rod, the end of which is bent at a right angle to the handle about one inch from the tip, streak the material over the surface of the plate in parallel strips as wide as the bent end of the spreader. Without sterilizing the spreader, similarly streak the material still adhering to it successively over 3 additional plates. In this way a progressive dilution of the suspension is secured, the degree of dilution depending upon the diameter of the spreader. With some practice one can be certain of finding a suitable distribution of colonies on one of the plates. After incubation for 24 hours suspicious colonies should be fished and subcultured on Russell's medium, or a preliminary macroscopic agglutination test may be made by placing a small drop of a 1 to 10 dilution of immune serum on a slide, touch-

ing the colony with the tip of a platinum needle, and rubbing this up in the drop. A positive reaction is indicated by the immediate appearance of visible clumps. Such colonies must be subcultured, and the agglutination confirmed by more precise methods. Any of these media will give satisfactory results after some experience with them has been obtained, but the observer must be familiar with the appearance of the colonies of the organisms for which he is searching on the particular medium employed.

Special media for isolating cholera vibrios are described in that chapter.

#### Chemical Examination

The reaction of the faeces is normally slightly alkaline to litmus. A strongly alkaline reaction suggests faulty digestion of protein and putrefaction. An acid reaction suggests disturbed carbohydrate digestion and fermentation. The faeces often become frothy on standing.

Pancreatic Ferments.—Amylase and trypsin are normally present. If quantitative estimations are needed, it is better to make them on duodenal contents than on facces. Winslow's method for determination of amylase (diastase) in urine (p. 731) may be applied to facces. A filtered 10% extract of facces in 0.1% Na<sub>2</sub>CO<sub>3</sub> is employed, and the results expressed as cc. of starch solution digested by 1 Gm. of facces. On a general hospital diet and after MgSO<sub>4</sub> or castor oil McClure and Pratt give normal values as 0 to 5000. Ferments are reduced or absent in acute necrosis of the pancreas, and in cases of obstruction of the pancreatic duct; they are variable in chronic pancreatitis.

Bile Pigments. Schmidt's (Qualitative) Test.—Rub up a little faeces in a saturated solution of HgCl<sub>2</sub> in a porcelain dish. Observe after several hours. Urobilin, the normal pigment, gives a salmon pink color; bilirubin, a green.

Quantitative Determination.—Most of the methods for quantitative estimation of urobilin in the faeces are cumbersome and require considerable experience to insure reliable results. Such estimations are often of value, however, in the study of haemolytic anaemias. Normally there are marked daily fluctuations, so that the determination should be made on a specimen representing the excretion of several days. The method of Wilbur and Addis (1914) has been most frequently used, but the end point (extinction of spectroscopic bands) is notoriously inaccurate and there is no way of converting such dilution figures into mg. of urobilin excreted. These objections are met by the procedure of Elman and McMaster as slightly modified by Josephs for clinical use.

(1) Collect the faeces over a period of three or four days, mix thoroughly with water and mash through an ordinary kitchen strainer, using sufficient water so that a representative sample can be easily obtained (500 cc. to 1500 cc., according to the volume of the faeces). Measure the total volume of the suspension. (2) To a small measured sample (25 cc.) add three volumes of acid alcohol (1600 cc. of 95% alcohol, 25 cc. of concentrated HCl, and water to give a volume of 2500 cc.). Shake the mixture and let stand over night in the dark. (During this time the urobilinogen is extracted from the faecal particles and largely oxidized.) (3) Shake and to 10 cc. of this suspension add a drop of tincture of iodine (to complete oxidation), 0.5 Gm. of zinc acetate, and 10 cc. of a saturated solution of zinc acetate in 95% alcohol, freshly prepared about every three days. Filter until clear. (4) In one of two absolutely clean test tubes of equal calibre put 15 cc. of standard acriflavin solution (1-30,000,000, freshly diluted from a stock solution containing one part in 100,000 and kept in the dark). In the other tube put

15 cc. of diluting fluid (2000 cc. of 60% alcohol, 50 Gm. zinc acetate, 10 cc. concentrated HCl, filtered until clear). To this tube add measured amounts of the solution to be tested until the fluorescence in the two tubes match. (For ordinary amounts of urobilin this will be in the neighborhood of 0.3 cc.) The comparison should be made against a black background with the light passing through the tubes at right angles to the line of vision. One mg. of urobilin in 2000 cc. of diluent matches the standard acriflavin solution when freshly prepared, saturated zinc acetate solution is used.

Calculation: The degree of dilution of the faeces in the final solution divided by 2000 gives the total excretion of urobilin in mg. for the period represented by the faecal collection. Thus, if the volume of the original faecal suspension is 1000 cc. and if 0.3 cc. of the fluorescent solution is added to 15 cc. of diluent to match the standard'

$$\frac{1000 \times 4 \times 2 \times 15}{2000 \times 0.3} = 200 \text{ mg. of urobilin.}$$

The average normal daily excretion of urobilin is roughly parallel to the amount of circulating haemoglobin; in adults, about 0.2 mg. per gram of haemoglobin, assuming the blood volume to be 76 cc. per kilo. There are great individual variations. The excretion is decreased during diarrhoea and also somewhat during constipation. An increase to 2 or 3 times the average may occur in a variety of conditions and usually has little clinical significance. In haemolytic anaemias during the active stages the amount excreted may be 10 or 15 times the normal.

Urine, if it does not contain bile, may be treated like the filtrate in (4) above, first adding a drop of tincture of iodine. If bile is present the results are inaccurate. The amount excreted in the urine is of little importance as compared with that excreted in the faeces and should not be used to estimate the severity of a haemolytic process. The ratio between the excretion in the urine and that in the faeces can be used, however, as a test of liver function. If more than 10% of the urobilin excretion occurs in the urine, it is indicative of liver damage, even in the absence of bilirubinaemia.

Ordinarily there is no urobilin in the bile, but Elman and McMaster have shown that infection of the biliary passages may cause a conversion of bilirubin to urobilin. This suggests that the presence of urobilin in the bile may indicate an infection of the bile ducts or gall bladder. Thus far this possibility has not been tested clinically.

Occult Blood.—In all tests for occult blood, chemically clean glass ware must be used. The activity of the reagents must be checked frequently, with a 1% dilution of blood. A button of faeces should be removed from the center of the faecal mass, to avoid surface contamination with blood from the rectum.

Gregorson test.—(1) Smear a little faeces over a glass slide. (2) Add a few drops of 50% acetic acid, and (3) a powder containing 0.2 Gm. of barium peroxide and 0.025 Gm. benzidine. This may be put up individually in waxed papers, or mixed in bulk, stored in dark bottles, and the quantity estimated approximately on a spatula. Result: (1) A deep-blue color in 3 seconds is a strong reaction. (2) A pale-blue color within

#### EXAMINATION OF THE FAECES

12 to 15 seconds is a moderate reaction. (3) A pale-blue or green color within 60 seconds is a weak reaction. The reagents are adjusted to limit the delicacy of the reaction, so as usually to avoid false positive reactions, including those due to the constituents of an ordinary diet.

The former procedure of adding a saturated solution of benzidine in glacial acetic acid, and H<sub>2</sub>O<sub>2</sub> directly to the faeces gives many false or clinically misleading positive reactions, and only negative results are significant.

Benzidine-hydrogen peroxide test.—(1) Make a moderately thin suspension of faeces in 5 to 10 cc. of water, add an equal volume of ether, shake well, and discard the ether (to remove fat). (2) Acidify strongly with glacial acetic acid, and (4) again extract with an equal volume of ether (which takes up the blood pigment). A few drops of alcohol will facilitate the separation of the ether. (5) Prepare the reagent: equal parts of a saturated solution of benzidine in glacial acetic acid, and hydrogen peroxide. (6) Stratify the ether extract over the reagent, or drop a little of the extract and of the reagents on a filter paper. A positive reaction is indicated by the appearance within 1 minute of a greenish-blue, deep-blue, or bluish-black color at the line of contact. The intensity of the color and the rapidity of its appearance serve as a rough gauge of the strength of the reaction. Feeble or doubtful reactions are of no significance.

Most interfering substances are removed. The reaction is very delicate, however, and may be influenced by the diet. A positive reaction should be checked by an examination after the patient has been on a meat-free diet for 3 days. A reaction obtained by adding the faecal suspension directly to the reagent is much less reliable.

The orthotolidine test may be used (see Urine, p. 726). The tests using guaiac or aloin have been largely discarded, since they are much less delicate, and have no compensating advantages over benzidine.

Quantitative Determination of Fats.—Cammidge's method (1914) is recommended by Beaumont and Dodds.

A. Total fat.—Dry a few Gm. of faeces to constant weight over a water bath and then in a vacuum desiccator. Grind up thoroughly in a mortar. Transfer exactly 0.5 Gm. to a 50 cc. graduated extraction tube such as a Schmidt-Werner milk tube. Add 10 cc. of 30% HCl (to split the soaps) and heat for 15 minutes in a boiling water bath, mixing the contents several times by carefully rotating the tube. Cool, fill to the mark with ether, stopper tightly, and invert tube 50 times so as to mix thoroughly. Let stand upright until the ether separates. Pipette off just 20 cc. of the ether layer into a weighed dish, evaporate the ether, dry in a vacuum desiccator, and weigh. The difference (w) between this figure and the weight of the dish is the total fat in 20 cc. of the HCl added) + 20 cc. (the ether removed), or y + 10 cc., is the volume of ether in which the fat from 0.5 Gm. of faeces is dissolved. Hence:

% total fat = 
$$\frac{w \times (y + 10) \times 100}{20 \times 0.5}$$

B. Neutral fat and fatty acids (without the soaps).—To determine this fraction weigh out a second portion of 0.5 Gm. of dried faeces, add 10 cc. of distilled water instead of 30% HCl and proceed as in (A). Calculate the combined percentage in the same way.

C. Fatty acids.—To determine these separately, redissolve the residue in (B) in 20 cc. of ether, add a few drops of alcoholic phenolphthalein solution, and titrate with

N/10 alcoholic sodium hydroxide. The fatty acids are all estimated (arbitrarily) as stearic acid, which has an atomic weight of 284. Hence:

% fatty acids = 
$$\frac{\text{Vol. N/10 NaOH used} \times 0.0284 \times (y + 10) \times 100}{20 \times 0.5}$$

By subtracting this figure from the combined percentage in (B), the percent of neutral fat is obtained.

# The normal figures are given as:

Total fat	15% to 25%	ć
Neutral fat	1% to 29	70
Fatty acids	9% to 139	$7\epsilon$
Soaps	5% to 100	-

In pancreatic insufficiency the total fat is greatly increased (to 60% or 80%), and it is largely neutral fat. In obstructive jaundice, sprue, and other conditions in which absorption is disturbed, there is also an excess of total fat in the faeces, but this is made up largely of fatty acids, since the lipase of the pancreas is not reduced in these conditions.

# CHAPTER XXXVII

# EXAMINATION OF THE GASTRIC CONTENTS

The examination of the gastric contents is a useful procedure in the study of gastric function, even though the practical value of the information so obtained is distinctly limited, and its interpretation often difficult. By this means one may obtain evidence as to (1) the motility, and (2) the secretory capacity of the stomach. It is the only direct method of determining the latter. However, in a large majority of patients with complaints referable to the stomach, the discomforts are immediately dependent upon disturbances of motility rather than of secretion. Roentgenological examination, as a rule, is more valuable than gastric analysis in detecting motility disturbances, although it is more expensive, and requires considerable experience for accurate interpretation. Gastric analysis, however, often gives valuable confirmatory information, and occasionally is decisive when X-ray fails.

The points of chief importance to be determined by examination of the stomach contents are: (1) Evidence of stasis; an abnormal volume of fluid and food retention 12 hours after a stasis meal (important). (2) The time required for the stomach to empty itself after a standard test meal, or the volume remaining after a definite time interval. (3) The capacity to secrete HCl and enzymes (important). (4) The degree of acidity and the quantity of enzymes formed after a standard stimulus. (This is less important. So many variable factors influence acid secretion that only gross variations from normal have any significance.) (5) Presence of abnormal constituents (lactic acid, bacteria, pus, blood, etc.). (Important in selected cases.) (6) The degree of digestion of the food (of minor importance).

There is as yet no general agreement as to the best procedure for such an examination. Opinions differ as to the best meal to give, as to the type of tube to use, and as to the time at which the contents should be removed.

Examination of *vomitus* is much less satisfactory than that of material obtained through a tube because it is always mixed with saliva and mucus from the mouth, and often with regurgitated duodenal contents, which dilute the gastric contents and directly neutralize some of the acid. However, it should always be examined when available, if passage of a tube is not advisable. Passage of a tube is absolutely contraindicated

in the presence of an aneurysm of the aorta, oesophageal varices (cirrhosis), obstruction of the oesophagus (cancer, stricture), and usually with an oesophageal diverticulum, a bleeding ulcer, and in myocardial insufficiency, extreme hypertension, or coronary disease (angina).

Passage of the Tube.—The large, semi-rigid tubes formerly used cause so much retching and discomfort to the patient that they are now rarely employed for test meals. An additional disadvantage is that the stomach can not be emptied so completely with them as with a small tube. They must be used for gastric lavage in case of poisoning, or in case the lumen of the small tube becomes plugged with mucus or coarse food particles.

The Rehfuss tube or one of its modifications is used extensively in this country. The Ryle tube (with a closed tip weighted with lead) is much used in Great Britain. The patient (if able to do so) should sit upright in a chair with the head thrust slightly forward. The metal bulb is dropped behind the tongue, and the patient is instructed to swallow it, and to continue to swallow, as he sucks the tube into the mouth, until the 50 cm. mark reaches the teeth. It is often necessary to give a small (measured) amount of water with the tube.

We prefer to use a catheter type of duodenal tube with lateral openings. such as the Levin tube (No. 16), or Sawyer tube, or in cases offering difficulty, a Jutte tube, which is passed by means of a blunt-tipped wire stylet, and requires no active cooperation from the patient. The tube is moistened and chilled in ice water. The patient is instructed to keep the mouth open and to breath rapidly and deeply through the mouth to control retching. The tip of the tube is pushed into the pharynx, and the patient is told to swallow once or twice to direct the tip into the oesophagus. It can then usually be passed on into the stomach without further swallowing. If necessary the throat can be sprayed with 2% cocaine solution. If one nasal passage is free from obstruction, it may be cocainized and a No. 12 or 14 Levin tube passed with a minimum of discomfort, but this is not recommended as a routine procedure. The patient is instructed to expectorate (and not swallow) any saliva or mucus that accumulates in the mouth.

Test Meal.—A stasis meal should be given 12 hours before the test if stasis is suspected. It may consist of rice pudding containing raisins, or a charcoal powder may be administered. The test meal should be given in the morning, with the stomach "empty." The tube should be passed and the stomach completely emptied before the test meal is given. To do this effectively the aspiration should be carried out with the patient recumbent, prone, and in both lateral positions. The normal fasting stomach contains from 20 to 100 cc. (occasionally 150 cc.) of fluid (average 50 cc.). A larger volume of fluid indicates a gross disturbance of motility. The presence of appreciable amounts of food residue nearly always means organic pyloric obstruction (usually due to ulcer or cancer). The contents should be saved for the usual examinations later. Free HCl is normally present (circa 20°), but is often absent in patients who show normal amounts of acid after a test meal.

The Ewald breakfast is still widely used: 2 thin slices of dry bread or toast (without butter) (35 Gm.), well masticated, and 2 cups (250 cc.) of clear tea (or preferably water, either hot or cold). In place of the bread, one may give a shredded wheat biscuit. or 8 arrowroot biscuits (which are free from lactic acid). This may be eaten with the small tube in place if the patient prefers. The Boas meal (r pint of strained oat meal gruel) is also widely used. .(Ryle, "Gastric function in health and disease," Oxford Med. Pub., London, 1926.)

Many prefer an alcohol meal, 70 cc. of 7% alcohol, injected through the tube. Some give 300 cc. of 5% alcohol. The chief advantage is that the stomach contents are "cleaner," and less likely to obstruct the tube than if food is administered. All are about equally satisfactory as stimulants of gastric secretion. The alcohol meal leaves the stomach more rapidly than the Ewald meal. Bergheim, Rehfuss and Hawk showed that plain water is an efficient stimulus.

Removal of the contents.—Many still follow the original method of Ewald, removing the entire contents of the stomach one hour after the patient started to eat the meal.

Others prefer fractional removal. A small tube is passed 30 minutes after the meal, and is left in place. A small sample (5 cc. to 10 cc.) is withdrawn at 15 minute intervals until the stomach is empty, or until 8 to 10 samples have been obtained. The stomach is emptied completely at the last aspiration. The specimens are examined individually. The stomach, however, does not mix its contents thoroughly. If a series of samples are withdrawn in rapid succession, either with or without altering the position of the tube, the various fractions often show marked differences in the degree of acidity present. The results of a fractional removal may, therefore, be misleading, unless the entire stomach contents are thoroughly mixed by repeated aspiration and reinjection prior to each removal. If this precaution is taken the method gives more complete and precise information as to gastric function than does a single aspiration, and often shows free HCl in cases in which it was not present in the single (one hour) specimen. One may question, however, whether the practical value to the patient of the additional information obtained adequately repays the extra time and discomfort involved, except in selected cases. Bloomfield and Keefer (1926) have devised an elaborate procedure for estimating also the rate of secretion and discharge from the stomach.

Histamine given hypodermically furnishes the strongest known stimulus to gastric secretion, and is the crucial test in differentiating between "true" and "apparent" achlorhydria. It is not to be recommended as a routine procedure, but, should be used, as a rule, in all cases showing no free HCl by other methods, particularly in cases suspected of pernicious anaemia or idiopathic hypochromic anaemia. A small tube is passed (fasting), and the stomach is emptied, the tube being left in place. Instead, the test may be given at the conclusion of one of the meals previously described, if no free HCl has been secreted. Inject 0.3 mg. histamine (as histamine or ergamine phosphate) hypodermically. Nothing is given by mouth. Empty the stomach after 15 minutes, and again after 30 minutes. Occasionally a third specimen at 45 minutes is desirable. The dose of histamine originally advised (0.01 mg. per kilo) gives a somewhat greater response, but often causes disagreeable reactions, and is not without danger.

Bockus and others have shown that about 50% of the cases who show no free HCl after an ordinary Ewald meal will show it after a fractional analysis, and an additional 25% will show it after histamine injection. The normal range of acidity is from 40° to 140°, usually between 90° and 125°. Cases of benign gastric ulcer practically always show free HCl of 30° or higher. The normal volume in the 15 minute period varies from 35 cc. to 150 cc.

### Examination of Specimens

Gross Appearance.—(1) Measure and record the *volume*. Delayed emptying is indicated by a volume of more than 150 cc. in the fasting stomach, of more than 100 cc. one hour after an Ewald meal, or by the presence of food and appreciable amounts of fluid more than 2.5 hours after such a meal fractionally removed.

- (2) Note the color. A yellowish or greenish tint indicates regurgitated bile. Small amounts are normal. Streaks of fresh blood are usually due to trauma from the tube. A diffuse reddish or brownish color suggests gastric bleeding. The "coffee grounds" sediment composed of blood altered by prolonged stasis suggests cancer.
  - (3) A foul or rancid odor in the fasting contents suggests cancer.
- (4) Mucus is normally present in small amounts. Excessive amounts most often come from swallowed sputum or nasopharyngeal secretion. This is often frothy, floating on the surface of the fluid in tenacious masses, and contains squamous epithelium, pus cells, and other constituents of sputum. Mucus may be secreted by the stomach in large amounts, particularly in chronic gastritis. It usually contains swollen gastric (columnar) epithelial cells and a few pus cells.

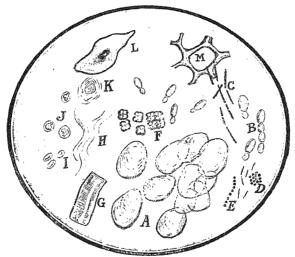


FIG. 184.—Microscopical constituents of the gastric contents. A, Starch cells; B, yeast cells; C, Oppler-Boas bacilli; D, staphylococci; E, streptococci; F, sarcinae; G, muscle fiber; H, mucus; I, red blood cells; J, leukocytes; K, snail-like mucus formations; L, squamous epithelial cells; M, cellulose. (From Hawk's "Physiological Chemistry.")

(5) Look for gross food residue in the fasting contents. Estimate roughly the amount of food residue and the degree of its digestion after a meal.

Microscopic examination of the residue from the fasting stomach is important, and must be made immediately. Examination of the sediment after a test meal rarely yields any useful information. Look for:

- (1) Food particles, particularly starch granules (test with iodine).
- (2) Blood or pus cells.—A few pus cells have no significance. Pus cells in appreciable quantity are nearly always swallowed (pyorrhoea alveolaris, chronic sinusitis or nasopharyngitis). Pus of local origin is seen infrequently in ulcerating cancer, and rarely in acute suppurative infections of the stomach wall.

- (3) Small fragments of tumor tissue may be found in very rare instances. Recognition is difficult.
- (4) Boas-Oppler bacilli; very large ( $1 \times 5$  to  $10\mu$ ), Gram-positive, non-spore-bearing organisms, growing in long chains and forming large masses. They are lactic acid producers and can be cultivated aerobically on media rich in milk or blood. They occur when stasis exists and HCl is absent, chiefly in cancer. They are significant only when present in large numbers.
- (5) Sarcinae in large numbers indicate stasis in the presence of HCl, and are seen most often in gastric ulcer with pyloric obstruction.
- (6) Parasites are rarely found. Flagellates have been found occasionally in cases of anacidity, as in some cases of early cancer.

#### Chemical Examination

Free HCl.—(1) As a quick, rough test, moisten a piece of *Congo red* paper with the stomach contents. An intense, deep-blue color indicates the presence of free mineral acid. A fainter dull-blue or violet may be due to organic acids.

(2) In a porcelain dish put a few drops of Boas' reagent (or Günzberg's), and evaporate to dryness (boiling water bath, or cautiously over a free flame; do not scorch). Add a drop of stomach contents and heat gently. The appearance of a bright rose-red color indicates the presence of free mineral acid. (The acid hydrolyzes the sugar, liberating levulose, which reacts with the resorcinol to produce the color: Seliwanoff's reaction). This test should be tried whenever a figure lower than 15° is obtained by titration with Töpfer's reagent, since the latter is not a dependable indicator of small amounts of free acid.

Boas' reagent: Resorcinol 5 Gm. Cane sugar 3 Gm. 50% alcohol 100 cc.

Günzberg's reagent: Phloroglucinol 2 Gm.; vanillin I Gm.; 95% alcohol 100 cc. This reagent gives a brighter color than the preceding, but is less stable.

(3) Quantitative estimation.—Filter some of the gastric contents through cheese cloth (or paper), and put a measured amount (10 cc. if available) in a porcelain dish. Add 3 drops of  $T\"{o}pfer's$  reagent. If free acid is present, the fluid turns a bright cherry-red color. From a burette add N/10 NaOH (N/100 if only 1 or 2 cc. of filtrate are available) until the color changes to a dull salmon-pink (Michaelis). Observe the volume of alkali used.

Calculation:

No. of cc. N/10 NaOH used × 100
Volume of sample titrated

Free HCl in "degrees of acidity,"

or "acidity per cent."

To convert this figure into absolute per cent of acid multiply by 0.00365.

Many titrate until a canary-yellow color is obtained. The color change is sharper, but the figures are too high.

Töpfer's reagent.—0.5 Gm. dimethyl-amino-azo-benzol in 100 cc. of 95% alcohol.

(4) Total Acid. (quantitative estimation).—To the same sample, after completing the preceding titration, add 3 drops of a 0.5% solution of phenolphthalein in 95% alcohol, and add N/10 NaOH from a burette until a deep red color is obtained. Observe the total amount of alkali added in both titrations. Calculation:

 $\frac{\text{Total No. cc. N/10 NaOH used} \times \text{100}}{\text{Volume of sample analyzed}} = \text{total acidity in "degrees."}$ 

The normal figures for an Ewald meal are from 25° to 50° free HCl, and from 50° to 80° (or even to 100°) total acid. The average normal figures for a fractional analysis are shown in the chart from Hawk.

(5) The difference between the "total acid" and the free HCl is commonly spoken of as the "combined acid." It includes the HCl combined with protein, the acid salts, and organic acids if present. Separate determination of these fractions is rarely of clinical value.

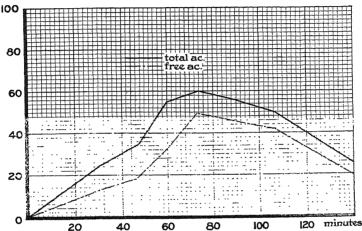


Fig. 185.—Acidity curves of normal human stomach. (From Hawk's "Practical Physiological Chemistry.")

(6) The "acid deficit" should be determined in specimens showing no free HCl. If the filtered juice gives a yellow color with Töpfer's reagent, add from a burette N/10 HCl until a bright red color is obtained.

This gives a rough idea of the degree of inadequacy of acid secretion. The gastric secretion is never alkaline to phenolphthalein. If alkaline material is aspirated, it indicates regurgitation of duodenal contents.

(7) The total chlorides in the gastric juice are sometimes a more reliable indicator of the secretory capacity of the stomach than is titration of the acidity. An unusual degree of regurgitation of duodenal contents may neutralize all the acid, even if this is secreted in normal amounts. Normally after a meal the total chlorides rise to about 500 mg. per 100 cc. of gastric contents, and may remain high for several hours. In achlorhydria they remain at about 200 to 250 mg. %. They usually parallel the curve

#### EXAMINATION OF THE GASTRIC CONTENTS

of free acid, and their determination is superfluous except in selected cases. They may be determined by the Volhard-Harvey method for chlorides in the urine if 10 cc. of juice are available, or by Whitehorn's method for blood chlorides, substituting 1 cc. of filtered gastric juice diluted to 10 cc. for 10 cc. of blood filtrate.

(8) The hydrogen ion concentration may be determined by the method described (p. 877) or more simply by the use of test papers (Töpfer's reagent and thymol blue), comparing the color obtained with standard charts. The additional information so obtained is rarely of much practical value.

(9) Lactic Acid. Kelling's test.—To a test tube of distilled water add 3 drops of 10% ferric chloride solution (fresh and light in color), and mix. Pour half into a similar tube as a control. Into one tube put a small amount of filtered gastric juice. Inspect by looking down into the tubes against a white background. A distinct canary-yellow color indicates the presence of lactic acid. Slight traces are of no significance.

Strauss' test.—Into a 25 cc. separatory funnel put 5 cc. of filtered gastric juice and fill to the mark with ether. Extract by inverting repeatedly for 10 minutes. Allow the ether to separate (adding a few drops of alcohol if necessary). Drain off and discard the watery portion, and add distilled water to the mark, and 2 drops of 10% ferric chloride solution. Mix gently. A strong greenish-yellow color in the water indicates the presence of at least 0.1% lactic acid.

Lactic acid (occasionally with acetic and butyric acid) occurs only when stasis exists in the absence of HCl, and strongly suggests cancer. It should be tested for in all cases with achlorhydria or marked subacidity.

(10) **Pepsin** is always present if HCl is secreted, and may be present in cases showing a complete achlorhydria. Clinically its estimation is of practical value only in such cases, in which it is desired to determine complete loss of gastric function (achylia gastrica).

Mette's method is the most practicable. Into each of 2 test tubes put 2 (better 4) Mette tubes and 15 cc. of N/20 HCl. To one tube add 1 cc. of filtered gastric juice diluted with 15 volumes of N/20 HCl. Incubate 24 hours at 37°C. Note whether the ends of the column of coagulated albumin in tubes in the diluted gastric juice have been digested, and if so measure accurately (in mm.) the length of the digested portion in each of the 4 (or 8) open ends, and take the average. The control tubes should show no digestion.

To express quantitatively in Mette units, square this average figure and multiply by 16 (the dilution). Normal figures are 2 to 4 mm. digested, or 64 to 256 units. Such figures are only approximations. Different specimens of albumin may show considerable differences in the amount digested when tested in the same specimen of gastric juice. If little or no digestion has occurred, the presence of small amounts of pepsin may be checked by repeating the test with higher concentrations of gastric juice.

To prepare the tubes, select capillary glass tubing with an internal diameter of 1 to 2 mm. Clean, break into 6 to 8 inch lengths, and fill (by suction or immersion) with clear, filtered egg albumin, free from air bubbles. Plug the ends with bread crumbs, and coagulate by immersion in a water bath at 85°C., leaving them in the water until the bath cools. Seal the ends with sealing wax, and store under water in the ice box. When needed, break the tubes squarely into sections about 2 cm. long, and discard any portions the lumen of which is not completely filled, or which contain air bubbles.

Trypsin (from regurgitated duodenal contents) may be determined by Spencer's method (1915). Its determination is rarely of practical value.

*Blood* should be tested for by the benzidine or orthotolidine method (see *Faeces*). Traces are of no significance (trauma of the tube). Bleeding in ulcer and cancer can be followed more conveniently by examination of the faeces.

Bacteriological examination of stomach contents rarely yields information of practical value. Normally they are sterile. If achlorhydria is present, the usual mouth flora are obtained. Tubercle bacilli can frequently be demonstrated in swallowed sputum from patients who fail to expectorate.

Interpretation of the results of these examinations requires much judgment and discrimination. In an untrained patient the results of the first examination are often misleading. Unexpected or bizarre results should always be checked. In practically all cases a definite diagnosis can be reached only in conjunction with the history and other findings.

In a majority of patients with gastric complaints (other than those due to gross dietary indiscretions) the symptoms are due to functional disorders brought about by disturbances of innervation, and not by organic disease in the stomach itself. Both motility and secretion are under nervous control. These disturbances may arise as a "reflex" from organic disease elsewhere, such as chronic appendicitis, cholecystitis, ureteral stricture, or pelvic disorders. In many cases they are simply a result of abnormal nervous tension, or of purely psychoneurotic disturbances. There is no finding characteristic of purely functional disorders. Pylorospasm and hyperacidity are common, but atony and subacidity occur. A great variation in the results of successive examinations is a suggestive finding.

Delayed motility may be due to (1) organic pyloric obstruction (cancer, ulcer with stricture, adhesions); (2) pylorospasm (functional or reflex disturbances of innervation, ulcer); (3) atony (most often seen in undernourished, asthenic individuals with visceroptosis, or as a purely functional disturbance).

Rapid emptying of the stomach is met with chiefly in (1) achlorhydria; (2) functional disturbances; (3) duodenal ulcer; (4) rarely in cancer infiltrating but not obstructing the pylorus.

Hyperacidity.—It has been shown by Boldyreff and others that normally pure gastric juice contains 0.4% to 0.5% HCl, and that the usual concentration of 0.15% to 0.2% is the result of dilution and neutralization by ingested food and fluid and by regurgitated duodenal contents. Hyperacidity is often associated with pylorospasm, which retards or prevents such regurgitation. It further causes a prolonged retention of food residue which stimulates a protracted and perhaps accelerated rate of acid secretion. There is often a progressive rise in acidity throughout

the fractional analysis. Hyperacidity is met with in many cases of gastric ulcer and most cases of duodenal ulcer, but is a common result of functional or reflex disturbances.

Achlorhydria of a temporary character occurs as a functional disturbance, and often in fevers and in debilitated individuals. True achlorhydria (or a marked subacidity) occurs (1) as a constitutional defect (often symptomless); (2) in many elderly individuals (a senile degenerative process); (3) in chronic gastritis (due to alcohol, faulty habits of eating, chronic focal or systemic infections, etc.); (4) in pernicious anaemia and idiopathic hypochromic anaemia; (5) in some cases of chronic gall bladder disease: (6) in most cases of cancer except those arising in a chronic ulcer. The achlorhydria often antedates the cancer, and is not necessarily a direct result of the cancer. (See Bloomfield and Pollard, "Gastric Anacidity," etc., Macmillan, 1933.)

Achylia gastrica (with loss of ferments as well as acid) occurs in most cases of pernicious anaemia, and in a smaller proportion of the other conditions listed above.

Gastric ulcer may show a normal or an increased acidity (rarely a subacidity), often pylorospasm and delayed emptying, and the intermittent presence of blood. These findings change but little if cancer develops in the ulcer. A large volume of fasting contents and a total acidity over 70° suggests a chronic ulcer with obstruction.

Carcinoma in an advanced stage may often be recognized by demonstrating achylia or achlorhydria, evidences of obstruction (large volume of fluid with 12 hour food-retention, lactic acid, Boas-Oppler bacilli), and blood (present in a majority of the cases). In the early stages a diagnosis can rarely be made by gastric analysis alone.

# CHAPTER XXXVIII

# EXAMINATION OF THE DUODENAL CONTENTS AND THE BILE

THESE examinations are so tedious and so uncomfortable to the patient and the information they yield is usually so meagre that they are indicated only in selected cases. In these few cases they may be of great value. Practically, examination of the duodenal contents is useful chiefly as a means of measuring the pancreatic ferments in suspected pancreatic disease. Examination of the bile gives positive information in some cases of cholecystitis and cholelithiasis, but in many cases no definite conclusions can be drawn. Much experience and critical judgment are required for the interpretation of the findings, and these must always be considered in conjunction with all the information obtainable by other methods of examination.

Procedure.—The examination is made in the morning with the patient fasting. A duodenal tube is passed to the 55 cm. mark, as previously described, and the stomach is completely emptied by aspiration and lavage. The patient then lies on the right side with the hips elevated and the tube held loosely in the mouth. By taking a few swallows of water and sucking in about 1 inch of the tube every 5 minutes (up to about the 75 cm. mark), the latter will usually enter the duodenum within 30 minutes to an hour. Pylorospasm may be overcome by injecting hot water through the tube, or by giving I mg. of atropin sulphate. Attempts to hurry the passage of the tube frequently result in its coiling up in the stomach. It must then be withdrawn to the 55 cm. mark, and the process repeated more gradually. The tube should be filled with water, and the drainage obtained by siphonage collected in small flasks or bottles, using a new bottle whenever the appearance or color of the fluid changes. The arrival of the tube in the doubt, this can be verified by the "duodenal tug," a prompt collapse of the tube when suction is exerted; and by the failure to recover promptly, by aspiration, water or colored fluid which is swallowed.

Normal duodenal contents consist of a thick, viscid, ropy fluid, clear or faintly opalescent, pearly-grey or light-yellow (bile), and alkaline to litmus unless mixed with gastric juice. It is a mixture in varying proportions, of duodenal secretion, pancreatic juice, bile, and gastric juice. Little or no useful information is obtained by titration or microscopic examination.

Pancreatic ferments may be measured by the method of McClure et al. (1921). The following simpler methods suffice for all practical purposes. Only gross variations from normal are significant. Estimations in duodenal contents are more reliable than in faeces. The fluid should first be made 8° alkaline to phenolphthalein with NaOH.

Trypsin.—We employ Gross' method in the following manner: In a series of six small test tubes, place 1.0, 0.5, 0.25, 0.1, 0.05 and 0.01 cc. alkalinized duodenal contents. To each add 2.5 cc. 0.1% casein in 0.1% Na<sub>2</sub>CO<sub>3</sub>, and mix. Add a 7th tube for control, containing only casein solution. Digest in water bath at 38°C. for fifteen minutes. Add a few cubic centimeters cold H<sub>2</sub>O; then acidify each tube with dilute acetic acid, and mix. Note least amount of duodenal contents producing complete digestion as indicated by transparency of the mixture, and report as number of cubic centimeters of casein solution that 1 cc. duodenal contents will digest. If this is the tube containing 1 cc. duodenal contents, report would be 2.5; if one containing 0.1 cc., 25, etc. We consider the normal to be 25-50.

Amylase (Amylopsin).—We apply Wohlgemuth's method in the same manner as in the previous test, using 1% soluble starch solution instead of casein, and digesting for thirty minutes. Test for starch with a few drops N/20 iodine in each tube. The tube with the least amount of duodenal contents, showing no blue color when mixed, is determined, and the result calculated as for trypsin. We consider the normal to be 2.5-5.0.

Lipase (Steapsin).—Place I cc. alkalinized duodenal contents in each of two large test tubes. Boil contents of one, and cool. To each tube then add I cc. ethyl butyrate, I cc. toluene. Shake well, and incubate at 37°C. for twenty-four hours, shaking several times in the interval. Then titrate acidity, using N/10 NaOH and phenolphthalein. The difference (in cubic centimeters) between the results of the two titrations represents the lipase. We consider the normal to be 0.2-2.0.

Parasites have been found in rare instances: Giardia lamblia, Strongyloides stereoralis, Endamoeba histolytica, 2-celled hookworm ova.

Cultures are of practical value only when the contents contain infected bile. Normally they are sterile. If achlorhydria exists, mouth organisms may be obtained. Regurgitation of colon bacilli and other intestinal organisms is rare.

Bile.—After sufficient duodenal contents have been collected for examination. slowly inject 50 cc. of hot 25% solution of magnesium sulphate through the tube. (If purging is contraindicated, hot bouillon or hot water usually suffice. Dilute HCl is an efficient stimulus, but it precipitates the fats and makes the specimens useless for examination.) Siphon (or if necessary aspirate) out as much of the solution as possible and collect the ensuing drainage in small bottles. To get satisfactory specimens it is necessary to watch the drainage constantly, and to change the container with each change in the appearance of fluid. As a rule, after from 5 to 30 minutes bile appears. If the duodenal contents were colorless, the first few cc. are usually bright light-vellow in color (Lyon's "A bile," which he believes comes from the ducts). This is quickly followed by darker bile, "B bile," which undoubtedly comes from the gall bladder, but is inevitably diluted more or less with duodenal secretion and pancreatic juice, fraction usually comes out rapidly in spurts, until from 5 to 50 cc. (or even 100 cc.) have been obtained. Normally this is deep golden-yellow in color, viscid and ropy, clear, and alkaline to litmus. This is often interrupted by spurts of cloudy, opaque, yellow ("egg-yolk") fluid, owing to an admixture of gastric juice. (If this is allowed to mix with the clear fractions, the specimen will be ruined.)

If no "B bile" is obtained within 30 minutes, a second and even a third injection should be tried. Flow may sometimes be stimulated by abdominal massage, or by having the patient walk about.

The B bile is followed by the slow, protracted drainage of a lemon-yellow fluid, "C bile," which Lyon believes comes directly from the liver.

Failure to obtain B bile may be due to: (1) Premature discharge from the gall bladder, before the tube is in place. (2) Spasm of the sphincter muscle (probably). (3) Obstruction of the cystic dust by stone or adhesions. (4) Failure of the gall bladder to fill and contract in a normal manner, usually due to cholecystitis. Total absence of pigments indicates complete obstruction of the common duct.

The color of the B bile may be abnormally dark; a golden-brown to a greenish-black. Such fluids darken rapidly and turn greenish on standing. This indicates stasis in the gall bladder, a condition which predisposes to stone formation. The fluid may be cloudy and contain considerable mucus in cases of chloecystitis. (Exclude acid from the gastric juice, and swallowed sputum.)

Microscopic examination of the centrifugalized sediment must be made immediately. Look for: (1) Pus cells which are bile-stained and scattered through the specimen in fair numbers point strongly to a cholecystitis. Unstained, "cleared" pus cells, and masses of mucus containing many pus cells are usually from the mouth. (2) Bile-stained epithelial cells and granular, cellular detritus suggest chronic cholecystitis. (3) Cholesterin crystals, as translucent or opaque, impure, flat, rhombic plates, often in irregular masses. (4) Calcium bilirubinate, in the form of light brown granules or dark redbrown or blackish precipitate. Large amounts of both these substances are highly suggestive (90%) of gall stones. Either or both in small amounts have no significance. (5) Rarely, microscopic gall stones ("bile sand"), as small, concentrically laminated concretions. Fatty acid and soap crystals often separate out on standing, but have no significance.

Negative findings are not conclusive.

Cultures of B bile from cases with active cholecystitis not infrequently reveal the causative organism, but the results of cultures must be interpreted with caution, particularly if achlorhydria is present. We spread the material on plain agar and blood agar plates, using r loop of material in one series, and r cc. in a second series. Cultures from the fasting stomach (and from the duodenal contents, if free from bile) are useful as controls. Perfect asepsis is impossible, and a few colonies must be disregarded, although normal bile usually yields entirely sterile cultures. A fairly abundant growth of a possible pathogen in relatively pure culture is usually significant, particularly if the control cultures do not show them. Colon bacilli are most often obtained, occasionally staphylococci, or streptococci. Typhoid bacilli can usually be obtained in culture from carriers, more easily and more regularly than from the faeces.

Systematic drainages have definite therapeutic value in selected cases, but the procedure has been much exploited and abused.

## CHAPTER XXXIX

## TESTS OF LIVER FUNCTION

THE liver performs, or participates in the performance of, many different and apparently unrelated functions of vital importance. Among others it brings about (1) de-aminization of amino-acids and synthesis of urea; (2) conversion of glucose, fructose, galactose, and lactic acid into glycogen, and storage of glycogen; and (3) mobilization and delivery of glycogen as glucose to the blood, so as to maintain approximately a constant blood-sugar level; (4) it takes up bilirubin (and urobilinogen) from the plasma and excretes it in the bile; (5) it synthesizes and excretes bile salts: (6) it excretes cholesterol; (7) it detoxifies many substances, both extraneous poisons (e.g. strychnine, cinchophen) and protein cleavage products of digestion; (8) it excretes certain dyes; (9) in most mammals except man it oxidizes uric acid; (10) it forms fibrinogen and probably other plasma proteins; and (11) through its Kupffer cells it participates in all the manifold functions of the reticulo-endothelial system, including the removal of bacteria and other particulate matter from the blood and the synthesis of bilirubin from haemoglobin.

Many different tests of liver function have been devised, based on these activities, but none has proved satisfactory as a means of detecting early or slight impairment of function. Many have been abandoned because experience has shown that they are unreliable or too insensitive to be of any practical value.

It is obvious that no single test can measure adequately the capacity of an organ with so many different functions. These functions in diseases of the liver are not depressed uniformly, nor is there any constant relation in the degree to which the different activities are depressed. It is not possible to associate any type of liver lesion with any one disturbance of function. Some of them are so much influenced by the activities of other organs that they are unreliable as an indication of liver injury (e.g., the storage and mobilization of glucose is influenced by insulin and epinephrin). Furthermore, the reserve capacity of the liver is so great, from 75 to 95 per cent for most of its activities, according to animal experiments, that its function is measurably altered only if the injury is diffuse and severe, and some functions (e.g., synthesis of urea) are maintained until destruction is practically complete. Focal lesions, even if extensive, rarely affect the usual tests appreciably, and even in cases of extensive cirrhosis compensatory hyperplasia may be adequate to maintain normal function according to the tests.

It follows that one should not depend upon any single functional test, but if possible several should be used. A normal result with any or all the tests does not exclude disease of the liver. On the other hand, if errors in technique can be excluded, a definite impairment of function revealed by any one reliable test is positive evidence of disease of the liver. Although in a very general way the more severe the liver injury is, the more impairment the functional tests will show, there is no close correlation between the degree of impairment and the gravity of the disease. However, a progressive improvement or deterioration of function, as shown by repeated tests on the same individual, has definite prognostic value. For bibliography and full discussion see review of Soffer (Medicine, 1935).

## DISTURBANCES OF BILE EXCRETION

These are characterized by hyperbilirubinaemia. Bilirubin is derived largely if not entirely from the haemoglobin of disintegrated red blood cells (and possibly from muscle haemoglobin). The process is discussed in the section on Haemolytic Anaemias (p. 350). Hyperbilirubinaemia is met with clinically: (1) in mechanical obstruction of the bile ducts; (2) in accelerated blood destruction; (3) in diffuse liver injury. In the latter group it is believed that two factors play a part: (1) the damaged liver is unable to remove bilirubin from the plasma as fast as it is formed, even though there is no pathological acceleration of haemolysis; and (2) if damage is intense and necrosis of liver cells extensive, the bile canaliculi probably become blocked, and the bile which has been secreted is reabsorbed, creating in effect an intrahepatic obstructive jaundice. It is obvious that mechanical obstruction of the ducts may lead to diffuse liver injury, particularly if associated with infection. It is probable also that some degree of liver injury must be present to produce jaundice in haemolytic anaemias, since 95% of a normal dog's liver may be excised without producing jaundice. This may be due to anoxaemia resulting from the anaemia (Rich).

Bilirubin which has been excreted by the liver and reabsorbed appears to differ in two important respects from that which has not passed through the liver cells, in that it gives a positive direct van den Bergh reaction and is readily excreted by the kidney, whereas the latter, which is believed to be combined in some way with plasma protein (or present in the form of a colloid, instead of a crystalloid), gives only an indirect reaction and is not excreted in the urine unless the concentration in the plasma becomes very high. A "biphasic" reaction is explained by the simultaneous presence of both types of bilirubin. If these views are valid, the otherwise puzzling behavior of the van den Bergh reaction, therefore, detects and differentiates between "free" (crystalloid) and "combined" (colloid) bilirubin and thus yields information of great diagnostic value. The quantitative van den Bergh measures the total bilirubin (of both types) present.

Van den Bergh Test: Qualitative.—(1) In each of 3 small tubes put 0.25 cc. of clear serum or plasma (which must be free from haemoglobin). The serum must be examined within 2 hours or kept on ice. (2) To the first tube add 0.2 cc. of water, and to the second tube add 0.2 cc. of freshly mixed diazo reagent, shake, and wait 5 to 10 minutes. (3) If any color appears add 0.2 cc. of diazo reagent to the third tube and observe constantly, noting when the color first appears, and when it becomes maximal (use the first two tubes for comparison). The development of the maximal color in tube 2 can be hastened by adding a crystal of caffeine-sodium-salicylate. (4) If no color appears in 5 minutes add 0.5 cc. of 95% alcohol, shake, and note whether any color has appeared. Centrifugalize if necessary.

The reaction can be made more sensitive by layering the reagent over the serum and observing the color at the plane of contact.

Interpretation.—An immediate direct reaction is indicated by the prompt appearance of a purplish-red color which becomes maximal within 30 seconds. A biphasic reaction is indicated by the appearance within 30 seconds (prompt biphasic) or 60 seconds (delayed biphasic) of the red color, which gradually increases in intensity. A reaction which does not appear until more than a minute has elapsed has about the same significance as a positive indirect reaction.

If no color appears within one minute, but does appear (promptly) after adding alcohol, this constitutes a positive indirect reaction. Any serum giving a positive direct reaction will also give a positive indirect reaction.

Quantitative Test.—(1) In a graduated centrifuge tube put 1 cc. of clear serum.
(2) Add 0.5 cc. of diazo reagent and shake.
(3) Add 2.5 cc. of 95% alcohol and shake.

(4) Add I cc. of saturated ammonium sulphate solution, mix, and let stand I5 minutes.

(5) Centrifugalize until clear. (6) Compare with standard solution in a colorimeter or comparator.

Calculation:

 $\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times_4 \text{ (dilution)} \times_{0.5} = \text{mg. bilirubin per 100 cc.}$ 

Some prefer to substitute for 4 in this formula the observed volume of the clear alcoholic solution above the precipitate in the centrifuge tube.

If a dilution type of colorimeter is used, the formula becomes:

Volume of unknown  $\times$  4  $\times$  0.5 = mg. per 100 cc.

Normal serum contains usually from 0.1 to 0.3 (never over 0.5) mg. per 100 cc. Van den Bergh's "unit" corresponds to 0.5 mg. per 100 cc.

Reagents. Ehrlich's diazo reagent. Solution 1.—Sulphanilic acid 1.0 Gm.; Concentrated HCl 10 cc.; water to 200 cc.

Solution 2.—Sodium nitrite 0.5 Gm. in 100 cc. of water.

Immediately before use add 0.1 cc. of solution (2) to 10 cc. of solution (1).

Standard solution.—Dissolve 2.161 Gm. of anhydrous cobalt sulphate (or if this is not obtainable, 3.92 Gm. of the recrystallized salt) in 100 cc. of water to which 0.5 cc. of concentrated sulphuric acid has been added. This keeps indefinitely in the dark. It corresponds in color to 0.5 mg. of bilirubin in 100 cc.

Significance.—In obstructive jaundice a positive direct reaction is obtained (occasionally with some "biphasic" intensification). A positive direct reaction may be given even by a trace of uncombined bilirubin, insufficient to raise the icterus index significantly. In haemolytic anaemias an indirect reaction is usual. In diffuse disease of the liver any type of reaction may be obtained, depending upon the stage and severity of the process. Thus in catarrhal and arsphenamine jaundice the reaction is at first indirect; later it becomes biphasic; and if necrosis is extensive it may become direct. If recovery occurs, the same changes occur in reverse order, and this may be the first favorable prognostic sign. The reaction is, therefore, not of help in distinguishing jaundice due to mechanical obstruction of the ducts from other types. It is not a sensitive indicator of liver injury, since many cases occur without hyperbilirubinaemia.

Icterus Index.—This measures the intensity of the yellow color of the serum. As this color depends largely upon bilirubin, the test is of some practical value in giving a simple approximate estimation of the bilirubin present. Haemoglobin, haematin, carotin and other lipochromes, if present, cause gross errors. The yellow pigments can be avoided by using a fasting blood specimen. Normally, however, the serum bilirubin is higher in the fasting specimen than after a meal. A normal icterus index excludes hyperbilirubinaemia. However, high values are much less dependable and should always be confirmed by the van den Bergh test. It is useful chiefly in following the course of a case from day to day.

Procedure.—Procure clear serum or plasma without the slightest trace of haemoglobin (the chief source of error). Put 1 cc. of serum in a comparator tube, and dilute with 0.85% salt solution until the color matches that of a 1–10,000 solution of potassium bichromate (to which  $\rm H_2SO_4$  has been added in the proportion of 4 drops to 1 liter, as a preservative). The volume of dilute serum in cc. gives the icterus index. Attempts to get more exact readings with a colorimeter are useless and imply a degree of precision which the test does not possess.

Significance.—Normal serum gives readings from 4 to 7 (maximum of 9). Readings from 10 to 16 may indicate "latent jaundice," while higher readings are obtained with evident jaundice (up to 100 or more). There is only an approximate correlation between these figures and those of the van den Bergh test. The icterus index figures may vary from 6 to 25 (usually about 10) times those of the van den Bergh test (expressed in mg. per 100 cc.). This discrepancy is due in part to the fact that the same amount of bilirubin in the uncombined (crystalloid) form imparts more color to a solution than it does in the combined (colloid) form.

Urobilinogenuria.—An increased excretion of urobilinogen in the urine has been utilized as a test of liver function. Normally the greater part of the pigment which is absorbed from the intestine is taken up by the liver and re-excreted in the bile as bilirubin, only traces reaching the kidney. Marked increases are frequently seen in haemolytic anaemias and in some cases of diffuse liver injury. However, experience has shown that this is not an early sign of liver injury and is often negative in cases with advanced injury. It has been found increased in about 20% to 25% of miscellaneous cases of definite liver disease. An increase in the proportion of the total urobilin excreted which appears in the urine is more significant (see p. 747). In complete obstructive jaundice the traces normally present disappear (except with acute infections of the bile passages). (For methods see p. 726, Examination of the Urine.)

Bromsulphalein Dye Excretion Test.—(Phenoltetrabromphthalein sodium sulphonate). This dye has largely supplanted those previously used and is, on the whole, the most satisfactory. Neither this nor any other excretion test is of any significance in cases with obstructive jaundice (when the serum yields either a direct or biphasic van den Bergh reaction). The dye may be purchased in 5% solution in ampoules ready for use (and also a comparator with standard color tubes) from Hynson, Westcott and Dunning, Baltimore).

Procedure.—(1) Weigh the patient, and calculate the number of cc. required to furnish a dose of 5 mg. per kilo (divide the weight in kilos by 10, or in pounds by 22). The dose of 2 mg. per kilo originally advised is much less efficient as a test. (2) Inject this quantity, undiluted, into a vein, slowly (1 minute), and carefully to avoid leakage. (3) At precise intervals of 5 minutes, 30 minutes, and 60 minutes withdraw 5 cc. of blood from the opposite arm vein, taking the usual precautions to prevent haemolysis. The 30 minute specimen is the significant one, and the others may be omitted in ordinary routine tests. (4) Put about 1 cc. of clear serum in each of two comparator tubes. To one add 1 or 2 drops of 10% NaOH to secure maximum depth of color, and to the other 1 drop of 5% HCl. (5) Read at once in the comparator, backing the standard tube with the tube containing acidified serum, and the alkalinized serum tube with a tube of water

Standard solutions may be prepared (in advance) by adding 4 mg. of the dye (0.08 cc. of the 5% solution) to 100 cc. of water which has been alkalinized with 0.25 cc. of 10% NaOH. From this solution, which corresponds to 100%, make progressive dilutions with similarly alkalinized water (80, 70, 60, 50, 40, 30, 20, 15, 10, 5%). Readings must be made promptly after the serum has been alkalinized, as clouding may occur after a few minutes and spoil the readings.

Interpretation.—The normal reading 5 minutes after injection is from 20% to 50% (average 35%). After 30 minutes all the dye, except perhaps an unreadable trace, has disappeared. The presence of ro% or more of the dye 30 minutes after the injection indicates definite impairment of function. In cases of grave liver injury considerable amounts may persist for several hours.

Significance.—The test is not a sensitive indicator of milder degrees of liver injury. Positive evidence of impairment has been obtained in about 60% of cases of proved liver disease, particularly in cirrhosis (but much less frequently if a dose of 2 mg. per kilo is used). This percentage is as large as, or larger than that given by any other test except the bilirubin excretion test. There is no close correlation between the amount of dye retained and the gravity of the disease, and a normal result does not exclude liver disease.

The rose bengal test (see Delprat and Stowe, J. Lab. and Clin. Med., 1931) has yielded about the same results as the preceding test, and we believe is in no way superior to it.

Bilirubin excretion test (von Bergmann).—This test has been improved and used extensively in the Johns Hopkins Hospital by Harrop and Barron (1931) and by Soffer and Paulson (1934). This procedure offers the obvious theoretical advantage that it tests the reserve capacity of the liver to perform one of its normal functions under an overload. It is useless and contraindicated in any patient with hyperbilirubinaemia (who gives a positive van den Bergh reaction, direct or indirect).

Procedure.—Bilirubin may be purchased from the Eastman Kodak Co. (at a cost of about \$2.50 for a single test). (1) Weigh out an amount of bilirubin equal to 1 mg. per kilo of body weight. (2) Dissolve this in 15 cc. of one tenth molar solution of sodium carbonate which has been boiled and cooled to 80°C. It forms a clear iodine-colored solution. (3) By venepuncture withdraw at least 5 cc. of blood and oxalate it; through the same needle inject the solution slowly, avoiding leakage. (4) After 5 minutes and again after 4 hours withdraw and oxalate at least 5 cc. of blood from the opposite arm. Avoid any haemolysis. (5) Precipitate the proteins by adding to 2 cc. of plasma from the first and the 4 hour specimens, 2 cc. of redistilled acetone; and to 1 cc. of plasma from the 5 minute specimen, 4 cc. of acetone. (6) Shake each, centrifugalize, and filter into a dry microcolorimeter cup. (7) Read, using as standard solution a 1-6000 solution of potassium bichromate.

Calculation: For the first (control) and 4 hour specimen:

mg. bilirubin per 100 cc. =  $0.329 \times 2$  (dilution)  $\times \frac{\text{Reading of standard}}{\text{Reading of unknown}}$ 

For the 5 minute specimen substitute 5 for 2 in the equation above.

(a) The difference between the 4 hour specimen and the control is the amount of pigment retained. (b) The difference between the 5 minute specimen and the control is taken as representing 100% of the pigment injected. (a) divided by (b) multiplied by 100 gives the per cent of bilirubin retained.

Significance.—In 28 normal individuals examined by these investigators the maximum retention after 4 hours was 5% to 6%, and in 15 all the pigment had been excreted. In 72 cases with definite but relatively mild liver injury, significant retention was observed in 86%, whereas of patients of this type only one fourth to one fifth as many showed retention of bromsulphalein. They found it by far the most sensitive indicator of slight impairments of liver function. Retention was observed in 16 of 29 cases after recovery from catarrhal jaundice (even 18 years after), and frequently in normal women

during the latter half of pregnancy. It, therefore, may be of less serious import than is evidence of impaired function by a less sensitive procedure.

### CARBOHYDRATE TOLERANCE TESTS

Galactose Tolerance Test.—Theoretically this sugar is an ideal test substance, since it is not utilized by muscle, and since normally all is taken up by the liver and converted into glycogen without need of insulin. Any that passes through the liver is quantitatively excreted by the kidneys, since the renal threshold for galactose is believed to be zero. It is not necessary to follow the blood sugar. The test may be used even in diabetics, and is especially useful in jaundiced patients.

Procedure.—Administer to the patient, fasting, 40 Gm. of chemically pure galactose in 250 cc. (or more) of water. Collect all the urine in separate hourly specimens for 5 hours, giving water as desired, but no food. Test each specimen for sugar (Benedict's method). Combine any specimens which contain sugar, and estimate it quantitatively, as glucose. If glucose is present it may be removed entirely, without appreciable loss of galactose, by rapid fermentation. Make up a 10% suspension of thoroughly washed yeast. To 7.7 parts of this add I part of urine and incubate for 45 minutes. Determine the quantity of sugar remaining, all galactose (Shay et al., 1931).

Significance.—A decreased tolerance is indicated by a total excretion of more than 3 Gm. of sugar. The view of Rowe that women normally have a higher tolerance than men is not generally accepted. The chief value of the test is in the differentiation of obstructive from non-obstructive jaundice. It is generally agreed that impaired tolerance is rare in the former group, but common in the latter (catarrhal jaundice, etc). In other types of liver disease the test is of little value, as it is rarely positive, and usually only in the advanced stages.

Levulose Tolerance Test.—This test is not applicable to diabetics, but may be used in jaundiced patients.

Procedure.—(1) Obtain blood from the fasting patient for a sugar determination.
(2) Administer 50 Gm. of pure levulose in 250 cc. of water (40 Gm. to patients weighing less than 140 pounds).
(3) Secure specimens of blood at half hour intervals for 2 hours, and make sugar determinations on each.

Based on the observations of various workers, the following may be accepted as criteria of impaired tolerance: (1) a maximum figure over 130 mg. (provided the fasting level is not over 115 mg.); (2) a rise of not less than 30 mg. if the fasting sugar is from 80 to 100 mg. (or from 35 to 40 mg. if the fasting level is less than 80); (3) failure to return to within 15 mg. of the fasting level after 2 hours.

Significance.—A significant impairment has been reported in about 50% of cases with well-marked liver disease, especially in cirrhosis. It is not of value in detecting slight degrees of injury but is nearly as sensitive as the bromsulphalein test, and yields positive results about twice as often as the galactose tolerance or urobilinogen test.

Other chemical changes may be observed in the most advanced (premortal) stages of liver necrosis:

- (1) Fall in blood urea N, with a rise in amino-acid N and total non-protein N.
- (2) Fall in urinary urea, with increase in ammonia and amino-acid excretion, and occasionally the appearance of leucin and tyrosin crystals in the sediment.
  - (3) Reduction of fibrinogen in the blood and a tendency to bleed.
  - (4) Marked hypoglycaemia.

The other tests of liver function are not considered because we regard them as either impracticable clinically, of doubtful reliability, or too limited in their application.

The mercuric chloride reaction of Takata and Ara.—This is not a test of liver function, but empirically it appears to be of some value in differentiating cirrhosis of the liver from other conditions clinically resembling it. It depends upon an increase in plasma globulin, with an inversion of the A/G ratio. The behavior of the test in other diseases showing an inverted ratio has not been adequately studied but positive reactions have been reported. Either serum or ascitic fluid may be used.

Procedure.—(1) Into each of a series of 8 small test tubes put 1 cc. of 0.9% salt solution. (2) Into the first tube put 1 cc. of serum, and prepare a series of dilutions by mixing and successively transferring 1 cc. of the mixture to the next tube. (3) Add to each tube 0.25 cc. of 10% sodium carbonate solution, and (4) 0.3 cc. of Takata's reagent. (5) Mix and observe immediately, after a half hour, and after 24 hours.

Takata's reagent.—To one part of 0.5% mercuric chloride solution add one part of 0.025% solution of aqueous fuchsin.

A positive reaction is indicated by definite precipitation in 2 of the first 3 tubes (regardless of what happens in the last tubes). Precipitation in the last 3 or 4 tubes only is of no significance.

The work of Jezler (1930), Crane (1934) and others indicates that the reaction is uniformly negative in normal individuals. It was positive in 90% to 95% of the cases of clinically well marked cirrhosis, and in several cases of acute yellow atrophy. It was negative in other types of liver disease. More study is required to establish the dependability of the test.

## CHAPTER XL

# FOCAL INFECTIONS

Chronic foci of infection are responsible for a number of systemic diseases that are grouped together under the term focal infections.

Focal infections are the result of a chronic rather than an acute focus of infection, and the bacteria concerned may be relatively avirulent. When highly virulent organisms overcome the local defense forces and become disseminated throughout the body from a local infection, an acute and usually fatal sepsis results. It is probable, however, that even in a healthy individual an occasional organism may gain entrance to the blood and tissues. In such a case the defensive forces of the body may dispose of the bacteria before any harm is done. Adami suggests that by such a process of "subinfection" an atrophy with replacement fibrosis may develop in various tissues of the body. These conditions, however, are not, strictly speaking, focal infections. A focus of infection in the body may affect the general resistance and predispose to, or aggravate, other diseases in an entirely non-specific way. In addition a lowering of the general resistance by any intercurrent disease may result in a flare up of some long standing local focus with dissemination of the bacteria or their toxic products into the general circulation.

Factors influencing the development of focal infections.—The precise manner in which bacteria in a chronic focus cause pathological changes in distant parts of the body depends upon various factors—the anatomical relationships of the nidus, the type of organism present, and the reaction of the various body tissues. The species of organism, and to some extent strain differences within a single species, play some part in determining the location and type of the secondary lesion. In some cases the organisms themselves are disseminated from the focus by way of the blood stream. If the resistance of a particular tissue is lowered by some preexisting disease or by trauma, the bacteria may gain a foot-hold in that area and cause a true metastatic infection. In some types of focal infection, however, bacteria cannot be demonstrated in the lesions, and it is difficult to trace the connection between the primary focus and the secondary lesion. It is possible that the pathological lesion is produced by a repeated lodging of small numbers of bacteria in a tissue, which eventually disposes of them, but becomes progressively injured in the process. On the other hand, one may equally well assume that the lesion is caused by the absorption of the toxic products of the bacteria in the primary focus. Again, some types of focal infection appear to depend upon the development of a hypersensitiveness to the protein of the bacteria in the primary focus. Some writers believe that local changes in the capillary circulation are of major importance in determining the development and location of the different focal infections.

The relationship between a chronic focus of infection and the various types of focal infection is, therefore, complex and in many cases controversial. A specific type of focal infection may occur only when several of these factors are present, and their occurrence and relative importance vary in the different types of focal infection.

Foci of infection.—In the study of all types of focal infection it is necessary to make a thorough search for every possible chronic focus. Frequently multiple foci are present, and it may be impossible to determine which one is the most significant. In some instances, particularly if the local lesion is long-standing, it is impossible to eradicate the infection completely. If the systemic condition has been present for a long time it may continue to progress after all foci have apparently been eliminated. In these cases it is assumed that the secondary lesion has become itself a focus from which in turn other areas may be affected. It is equally probable, however, that treatment of the original focus has not entirely eradicated the infection. The early recognition and treatment of all foci of infection is, therefore, of prime importance.

The relative importance of the different foci is difficult to evaluate. It is generally agreed, however, that infections in the tonsils and around the apices of the teeth are responsible for a great majority of the focal infections. In these deep hidden areas the anatomical conditions are such that the escape of the bacteria externally is prevented, and absorption takes place directly into the lymphatics and blood stream. It has been suggested that the stress to which these areas are subjected during mastication and deglutition facilitates dissemination of the infection. Chronic infections of the genito urinary tract, nasal sinuses, middle ear, bronchi, gall bladder and appendix may be responsible for a minority of the focal infections. The importance of the gastrointestinal tract as a focus is a subject of active controversy. That actual ulcerative lesions in the colon may act as foci can scarcely be questioned, but there is little convincing evidence that the normal colon can exert such an effect.

Focal infections.—This term embraces a number of diseases in various tissues which are believed to be caused directly or indirectly by a chronic focus of infection. The type of lesion produced varies greatly, and not infrequently more than one condition is present in the same individual.

In subacute bacterial endocarditis the bacteria themselves enter the circulation and localize on the cardiac valves, but as a rule, only when the valves have been injured previously. In this case, therefore, only two factors appear to be involved—an invasion

of the blood stream, and a lessened resistance of the tissues. (This condition is discussed more fully in the section on the streptococcus.)

Infectious (rheumatoid) arthritis is perhaps the commonest result of a chronic bacterial focus. The clinical and etiological classification of the various types of arthritis is still confused, but the dependence of some types upon a streptococcal focus cannot be doubted. Bacteriological studies of the blood and joints have yielded contradictory results. (See section on streptococcus.) The precise manner in which the joints are injured is, therefore, not clear. It is possible that a few streptococci may be carried repeatedly to the joints, where they may persist in small numbers or are eventually killed, but with each invasion, there is a progressive injury to the tissues. However, other factors certainly enter into the picture, particularly the development of a hypersensitiveness to the bacterial protein. Circulatory changes probably play some part, and possibly other factors.

Acute rheumatic fever is likewise dependent upon a focus of infection, usually in the throat. Bacteriological studies have given conflicting results, and the factors concerned are even more obscure than in rheumatoid arthritis.

A chronic gonococcus infection may cause an arthritis that may resemble the ordinary infectious type. The gonococcus may be isolated from the joint fluid in acute cases.

Glomerular nephritis is commonly associated with a beta (haemolytic) streptococcus infection, though the alpha types may be concerned in the less acute cases. It is now believed by many that the so-called lipoid nephrosis also depends upon bacterial infection. Blackman (1935) found chronic pneumococcal infections in several cases falling into this group, and produced a typical lipoid nephrosis in animals by small doses of pneumococcal toxin. It is probable that the type of renal lesion depends upon the species of bacteria concerned, and upon the chronicity of the focus.

Pyelonephritis, pyelitis, and ureteritis are believed to be due in some cases to a remote bacterial focus. The status of ureteral stricture is still disputed. Some investigators consider that the obstruction is due primarily to a localized metastatic infection in the ureter, whereas others consider that the condition is purely a localized spasm independent of a focus of infection.

Various inflammatory processes in the eye are caused by a focus of infection. Sensi tization of the various tissues in the eye is an important factor in these cases.

Gastric and duodenal ulcers have been attributed to a streptococcal focus, particularly by Rosenow and his followers. Although streptococci may play a part in their development, it has not been demonstrated that it is a fundamental one. There is no conclusive evidence at present that ulcerative lesions in the intestinal tract are true focal infections. Cholecystitis, cholangitis and hepatitis and pancreatitis may, in some cases, result from a distant focus.

Chronic urticaria in adults is frequently an allergic reaction to the proteins of the bacteria in a chronic focus. Other skin lesions may also be connected in some way with remote bacterial infection.

In addition to these conditions there are many others more or less clearly dependent upon foci of infection. The presence of such areas may cause vague disturbances of the general health, and predispose to the development of other unrelated diseases.

Bacteriological studies.—The organism most frequently concerned in the focal infections is the streptococcus, particularly the alpha (nonhaemolytic) type. These organisms are not, as a rule, very virulent. The beta (haemolytic) streptococcus is also very important. Occasionally other species of bacteria such as the pneumococcus, staphylococcus, gonococcus, and influenza bacillus give rise to true focal infections.

In most of the focal infections cultures from the secondarily affected areas and from the blood do not reveal organisms. (Exceptions to this rule are discussed in the section on the streptococcus.) If, however, one obtains pure cultures of some organism from a focus of infection, and if the systemic condition clears up after removal of the focus, it is reasonable to assume that the organism obtained was responsible in some way for the condition. This assumption is strengthened if an allergic reaction is obtained by a suitably controlled intradermal test.

When cultures are made from the oral cavity, it must be remembered that alpha streptococci of various types are almost invariably present, and beta streptococci not infrequently so, even in a healthy mouth. Both are frequently found in the gastro-intestinal tract, and their presence in a stool culture is not necessarily significant.

Leukocytosis or leukopenia with changes in the differential formula have been described as characteristic of a chronic bacterial focus. Abnormal counts may undoubtedly be caused by a focus, but they are not characteristic, and more frequently the count is normal. An abnormal count, however, for which no explanation can be found should suggest a thorough search for some hidden focus of infection.

Elective localization.—Rosenow and his associates, Haden, and others have shown that if an animal is injected intravenously with a culture of a streptococcus freshly isolated from a human focus, localized lesions may develop corresponding in type and location to those present in the patient. This does not invariably occur, however, and frequently the lesions produced are multiple. The following table compiled by Haden from a large series of animals illustrates the frequency with which the human lesion was reproduced, and also the occurrence of other lesions in the animals, particularly in the joints. Other investigators have been unable to confirm these results. Furthermore, intravenous injection into animals does not reproduce exactly the conditions existing in a patient with a long-standing localized bacterial focus from which remote lesions have developed.

Rosenow believes that the particular type of focal infection which results from a chronic streptococcal focus depends upon an "elective affinity" of that strain for some particular tissue. This concept is interesting, but it is not generally accepted. Others believe that the location and type of the focal infection depends upon the susceptibility of some particular tissue, or upon local changes in the capillary circulation.

At present one must conclude that the development of focal infections, and their location and type, depend upon a number of factors, and no single theory will explain them all. Particular bacterial affinities and tissue susceptibilities may both be important, but the other factors mentioned previously also play a part. The relative importance of each of

these probably varies in the diverse pathological processes which are included in the term focal infection.

Incidence of Lesions Produced in Rabbits by Intravenous Inoculation of Streptococci Cultivated from Dental Infections in Man Compared with the Systemic Lesions in the Case from Which Each Strain Was Derived (After Haden, from American Journal of Medical Sciences, Dec., 1926)

			Pe	rcent	age o	ge of animals showing lesions				
Cultures from patients with	No. of animals	No. of patients	Joint	Kidney	Muscle	Endocardium	Myocardium	Brain	Eye	Stomach and duodenum
Kidney infection	48	10	73	85	25	4	4	2	6	6
Heart and vascular disease	46	ıı	72	30	22	57	37	2	4	13
Eye infection	142	30	56	33	18	14	10	9	65	6
Peptic ulcer	109	25	53	23	29	14	12	2	5	57
No known kidney, eye, heart or gastric disease	1155	425	57	28	20	17	9	4	9	13
Total	1500	501								

## CHAPTER XLI

# THE ENDOCRINE GLANDS

In spite of the intensive work which has been devoted to the study of the endocrine glands and the progress which has been made in determining their functions, knowledge of the diseases of these organs is still fragmentary. Diagnosis is often based largely on purely clinical findings and is still confused by much ill-founded speculation and hypothesis. Much of our knowledge is derived from animal experiments, and the results of such studies can not necessarily be applied to man. There are few specific tests available which are dependable and practicable as diagnostic procedures.

In the following paragraphs an attempt is made to summarize very briefly the main facts and the prevailing opinions as to the most important functions of these organs. Some of these are still in controversy, and many of them will require revision as more knowledge is obtained. For a fuller discussion the reader is referred to the series of special articles appearing in the Journal of the American Medical Association, 1935, Feb. 9 to Aug. 31, (inc.), and to special monographs.

Pituitary, Anterior Lobe.—The anterior lobe of the pituitary gland has been shown to exert so many activities that it has been called "the general headquarters of the endocrine system." Evans recognizes at least five distinct hormones.

- 1. The growth hormone (antuitrin G), secreted by the eosinophilic cells, is indispensable for normal growth and development of young animals and probably plays a part in maintaining normal conditions in the adult. An excessive secretion in the young results (usually) in gigantism; in the adult, in acromegaly. A deficiency of the hormone in young animals results in various types of dwarfism. Injections of the hormone have stimulated growth in some human dwarfs.
- 2. Gonad-stimulating hormones, secreted by the basophilic cells, exert two distinct activities:
- a. The "follicle-stimulating" hormone causes the development of the Graafian follicles and excites the secretion by the latter of the oestrogenic hormone. In males it stimulates spermatogenesis. A deficiency results in failure of gonadal development and indirectly in sexual infantilism.

b. The "luteinizing hormone" causes the luteinization of the cells of the follicle and stimulates the formation by the corpora lutea of their special hormone, progestin. In the male it is thought chiefly to stimulate the interstitial cells of the testis.

Minute amounts of these substances (or of substances having similar activities) are found in the urine of normal women after puberty. In women after the menopause and in castrates, considerable amounts of the follicle-stimulating hormone (Prolan A of Zondek) are often found. In the urine of pregnant women there are also found large amounts of a substance resembling in its activities the luteinizing hormone of the hypophysis (Prolan B, antuitrin S, anterior pituitary-like substance). Its action differs in some important respects from that of anterior lobe extracts: e.g., it is inactive in hypophysectomized animals. It is probably formed in the placenta.

- 3. The *lactogenic hormone*, prolactin, excites alveolar hyperplasia and milk secretion, although it is not the primary stimulus to normal breast development. It has been suggested that an excessive secretion may stimulate the development of breast tumors.
- 4. The thyrotropic hormone stimulates the normal development of the thyroid gland. A deficiency results in atrophy of the thyroid with secondary myxoedema. It is not known whether or not an excess may cause thyroid hyperplasia and exophthalmic goiter.
- 5. The interrenotropic (adrenotropic) hormone stimulates the normal development of the adrenal cortex. An excess (in animals and presumably in man, in Cushing's syndrome) causes cortical hyperplasia. A deficiency might be expected to cause cortical atrophy (with the symptoms of Addison's disease).

By suitable methods extracts of each of these hormones may be obtained free or nearly free from the activities of the others. None of them have been identified chemically, except that they appear to be protein substances. It is not certain whether they are fundamentally different, or are related derivatives of some common mother substance.

Anterior lobe extracts also show (6) "diabetogenic" activity, causing hyperglycaemia, glycosuria and a "diabetic" type of response to glucose tolerance tests; and (7) a "ketogenic" activity, with a lowering of blood fat, and increase in the ketone bodies in the blood and urine. Collip believes that these two activities are due to distinct hormones. They are obviously antagonists of insulin. There is no proof, however, that they play a part in human diabetes. A deficiency results in hypoglycaemia and

increased sensitiveness to insulin, and lessens the severity of the diabetes in a depancreatized dog.

It is possible that there are other hormones that stimulate development of the parathyroids and the pancreatic islets; hyperplasia of these structures has been described following administration of anterior lobe substances.

The *adiposity* formerly attributed to hypophyseal insufficiency is now known to be due to injury to the infundibulum.

Pars intermedia. The pars intermedia of the hypophysis secretes a distinct hormone, *intermedin*, which causes an expansion of the melanophore cells in the frog's skin. It is present in man, but what function it fulfills is not known.

Posterior Lobe.—The posterior lobe secretes two hormones:

- I. *Pitocin* (oxytocin) stimulates contraction of the uterine muscle. Its effect is maximal at the time of labor. This augmentation is attributed to the synergistic action of oestrogenic hormone which is abundant in the blood at that time.
- 2. Pitressin (vasopressin) stimulates smooth muscle generally. It constricts the peripheral vessels and may raise blood pressure, increases peristalsis and lessens diuresis in diabetes insipidus and after copious water drinking. Both are antagonists of insulin and cause hyperglycaemia.

Thyroid.—The thyroid gland secretes an active substance, iodothyroglobulin, abundant in the colloid, of which thyroxin is believed to be a split product. The effects of thyroxin are very similar to those of thyroid substance. It increases the rate of oxidation, raising the basal metabolic rate, it increases the pulse rate and exerts an important influence on growth and sexual development. It acts as a synergist to epinephrin, increasing the pressor and hyperglycaemic effects of the latter, and is an antagonist of insulin. Excessive secretion occurs in exophthalmic goiter and toxic adenomata. A congenital deficiency results in cretinism, whereas a deficiency acquired later in life causes myxoedema. Theoretically the latter might be due either to a defect inherent in the thyroid itself, or to lack of the thyrotropic hormone of the pituitary. The disturbances caused by thyroid deficiency are not simply the result of a retarded rate of oxidation. Myxoedema is relieved by thyroid, but not by dinitrophenol which is equally effective in raising the basal metabolic rate.

The thyroid hormone appears to exert an inhibitory effect on secretion of the thyrotropic hormone, since thyroidectomy is followed by hyperplasia of the anterior pituitary with excessive production of this, as well as of other pituitary hormones. This antagonistic action is believed to result in an equilibrium which under normal conditions maintains thyroid secretion at an optimal level. Analogous interrelationships exist between the thyroid and other endocrine glands. Thus the ovary and the adrenal cortex appear to inhibit thyroid secretion, perhaps indirectly, through the inhibitory effect of the oestrogenic hormone on the anterior pituitary.

A deficient iodine intake results in marked thyroid disturbances which Marine believes are manifested first by hyperplasia, and later result in simple colloid goiter as recovery occurs. There is not as a rule a gross disturbance in the amount of hormone secreted, but there is a marked tendency to the development of adenomata and other abnormalities in such glands, and cretinism may develop.

Ovary.—The ovary secretes two important hormones:

- I. The *oestrogenic hormone*\* (oestrin, theelin or estrone, folliculin, etc.) is formed by the cells of the follicle, under the stimulus of the folliclestimulating hormone of the anterior pituitary. It stimulates the development of the secondary sexual characters, the growth of the mammary glands and nipple, and the development of the uterus and its appendages. It causes hyperplasia of the endometrium in the initial stages of the menstrual cycle, and proliferation and cornification of the vaginal epithelium which precedes oestrus (conspicuously in rodents, demonstrably in man). This forms the basis of the Allen-Doisy test for its detection and measurement. It increases libido. The quantity in the blood is increased during the initial phase of the menstrual cycle, falling abruptly as bleeding begins. It is increased in pregnancy after the second month, reaching a high level at labor and dropping abruptly within a few days after delivery. During pregnancy it is formed in large amounts by the placenta. 'It exerts an inhibitory effect on the formation of the follicle-stimulating hormone by the anterior pituitary. Some believe that fluctuations in the equilibrium between these opposing forces explain the rhythmical occurrence of the menstrual cycle. (For details as to the amounts of these hormones present and methods of titration see Frank et al., 1931, 1934, 1935).
- 2. Progestin (progesterone) is secreted by the cells of the corpora lutea under the stimulus of the luteinizing hormone of the anterior pituitary. Its principal function seems to be to bring about those changes in the

<sup>\*</sup> At least seven different oestrogenic substances, closely related chemically, have been isolated in crystalline form from the urine or body tissues. Little is yet known as to their relative importance. For approved nomenclature and structural formulae see Report of Council on Pharmacy and Chemistry, J.A.M.A., Oct. 10, 1936. The term "oestrin" is used in this discussion to include any or all of them.

endometrium (after if has become hyperplastic under the influence of oestrin) which make possible the implantation of the ovum and the development of the placenta. In this respect it is an antagonist of oestrin, although in some other respects they appear to be synergists. Some believe that a deficiency of progestin, with an excess of oestrin, may be one cause of early miscarriages and of pathological endometrial hyperplasia with metrorrhagia, and may contribute to the formation of myomata and other uterine tumors.

Neither of these hormones stimulates the ovary, but on the contrary through the pituitary they exert a definite inhibitory effect. Structural formulae have been worked out which indicate that the two hormones chemically are closely related to each other and to the male sex hormone, and also to some of the carcinogenic hydrocarbons.

Placenta.—The placenta, as noted, produces oestrin and the anterior pituitary-like hormones, both a luteinizing hormone (Prolan B) and a follicle-stimulating hormone. Collip believes the latter differs in some respects from that in pituitary extracts and has named it emmenin.

Testis.—The interstitial cells of the testis, under the stimulus of the gonad-stimulating hormone of the anterior pituitary, secrete the male sex hormone, androsteron. This stimulates the growth of the accessory sex organs and the development of the secondary sex characters. It prevents or corrects the involutional changes in animals which follow castration. It does not stimulate the testes, but, like oestrin, exerts an indirect inhibitory effect through the pituitary. There is no proof that it exerts any rejuvenating effect. It is found in the blood and urine of sexually mature male mammals during the periods of sexual activity (continuously in man and the common laboratory rodents, cyclically in most other mammals). It is commonly estimated by measuring the rate of growth of the comb of capons stimulated by it.

Adrenal.—The adrenal cortex secretes a hormone (interrenin, cortin), the primary function of which appears to be to regulate the metabolism of sodium and potassium. It is essential to life. Extracts have been prepared in research laboratories (but not as yet commercially) which maintain adrenalectomized dogs indefinitely in good health. Deficient secretion (as in Addison's disease) may be due to local disease of the adrenal (tuberculosis, tumors), or conceivably to lack of the adrenotropic hormone of the pituitary. The disturbances caused by a gross deficiency are numerous and characteristic; excessive excretion of sodium in the urine and a marked reduction of sodium, and of chloride, bicarbonate or

both in the blood; dehydration; fall in blood pressure; retarded basal metabolic rate; muscular weakness; anorexia and vomiting; and renal insufficiency with rise in blood urea, and finally anuria. There is hypoglycaemia and increased sensitiveness to insulin. Some but not all of these disturbances may be relieved simply by replenishing sodium chloride and water.

Excessive secretion, probably occurring in certain tumors of the adrenal cortex, causes obesity, hirsutism, amenorrheoa and other symptoms which may be practically identical with those of basophilic adenoma of the pituitary.

The adrenal medulla secretes epinephrin. The pharmacological actions of this drug are too well known to need discussion here, but the rôle which it actually plays in the body in normal or pathological states is still unknown. (Some of its functions are discussed in the section on Carbohydrate Metabolism.) The work of Rogoff and many others indicates that it is not essential to life and health and that it does not act. to maintain normal blood pressure. Although hypertension (often paroxvsmal) is a characteristic feature of certain tumors of the adrenal medulla, there is no convincing evidence that it is a cause of essential (or nephritic) hypertension, or of human diabetes. Cannon's view that epinephrin is secreted in emergencies, to put the animal in an optimum condition for flight or combat, is plausible, but this is questioned by Rogoff because the experiments on which it was based are open to serious objections. No available methods are specific and sufficiently sensitive to measure the minute amounts of epinephrin (one in a billion or less) which may be present in the peripheral blood.

Parathyroids.—The parathyroid glands secrete a hormone which controls the metabolism of calcium and phosphorus. It is thus concerned in the nutrition of bones and teeth, and regulates (lessens) the irritability of motor nerves and muscles. Excessive secretion (parathyroid adenomata) causes absorption of calcium and phosphorus from the bones and increases the excretion of these elements in the urine. The blood calcium is elevated and in marked cases the phosphorus also. There is rarefaction of the bones (osteitis fibrosa cystica), with secondary deposits of calcium salts in the soft tissues, the formation of renal calculi and consequent impairment of renal function. Muscular weakness becomes extreme. Deficient secretion causes a fall in blood calcium with a compensatory rise in phosphorus and the development of tetany.

Pancreas.—The action of insulin is discussed in the section on carbohydrate metabolism.

Thymus.—The thymus gland presumably exerts an influence on the growth of young animals, but very little is known as to its precise function or its hormone. Normally it undergoes atrophy when the period of growth ceases, probably under the influence of other endocrine glands (adrenal cortex, gonads, possibly the pituitary, although some have reported hyperplasia of the thymus after injections of anterior pituitary extracts). Hyperplasia of the thymus is common in exophthalmic goiter. Rowntree et al. have shown that repeated injections of thymus extracts over successive generations of rats result (after the second generation) in astonishing acceleration of the rate of growth and development of young animals without causing gigantism. These animals showed small adrenals and lymphoid hyperplasia, resembling to this extent human cases of status thymico-lymphaticus.

Antihormones.—Long continued injections of certain hormones into animals (e.g., the thyrotropic hormone) may result in the development in the animal of a refractory state. The serum of such animals has been found to neutralize or inhibit the effect of the corresponding hormone when injected into untreated animals. This effect is attributed by Collip to a specific antihormone. If this conception is correct, these antihormones obviously may play a very important part in the normal control of the activities of the endocrine glands. His explanation of the phenomenon has not as yet been accepted generally.

## BASAL METABOLISM

By basal metabolism is meant the total energy produced by the combustion of food substances in the body under conditions which reduce the energy expenditure to a minimum; i.e., when the individual is fasting and is physically and mentally at rest and relaxed. The metabolism is raised above the basal level by the chemical processes involved in digestion and absorption (10% to 15%) and by mental or physical activity.

The metabolism may be determined directly by measuring the heat liberated in a calorimeter, or it may be calculated if the oxygen consumed and the carbon dioxide liberated (and the N excreted in the urine) over a fixed period are measured accurately. The amount of heat liberated for each liter of oxygen consumed ("caloric value" of O) (or carbon dioxide produced) is not a constant but depends upon the relative amounts of carbohydrate, fat and protein in the food mixture oxidized. If carbohydrate only is burned, for each atom of C burned, one molecule of O is consumed and one molecule of  $CO_2$  is produced. The respiratory quotient (R.Q.) or the ratio by volume of the  $CO_2/O$  involved is, therefore, r.o. The caloric value of one liter of O (and also of  $CO_2$ ) under these circumstances is found by calculation and observation to be 5.05 calories. If fat only is burned, aside from the O required to oxidize

the C to CO<sub>2</sub>, additional O is needed to oxidize the H, which is eliminated as water. The ratio of CO<sub>2</sub>/O is therefore less than 1.0 (for average fat 0.707) and the caloric value of one liter of O is less than that of one liter of CO<sub>2</sub>. (O, 4.69 cal.; CO<sub>2</sub>, 6.63 cal.) Under all ordinary conditions fat and carbohydrate are both burned, in varying proportions, as well as some protein (which has an R.Q. of about 0.80). To estimate the energy output precisely in this way is a tedious procedure.

It has been found, however, that in the fasting state if the subject has been taking an average diet, the R.Q. is relatively constant, on the average 0.82, and that for routine diagnosis (except in diabetics of appreciable severity) no error of practical significance is introduced if this average figure is assumed. It is, therefore, possible to calculate the basal metabolism with sufficient accuracy by determining either the O consumption or the CO2 output. Both procedures have been employed. Calculations based on O consumption are more reliable than those based on CO2 excretion alone. The variation in the caloric value of CO2 is much greater than that of O if the actual R.Q. varies from the average figure. There may also be a "pumping out" of CO2 from the plasma bicarbonate if the pulmonary ventilation rate is increased (or a storage of CO2 if the rate is diminished) during the brief period of the test. The caloric value of a liter of O with a R.O. of 0.82 is 4.825 calories.

Methods.—If both O consumption and CO<sub>2</sub> elimination are to be measured and the R.Q. precisely calculated, the Tissot "open method" should be used. This is practicable only in well equipped laboratories with specially trained technicians, and the procedure will not be described.

For determinations of O consumption alone, several satisfactory simple "closed circuit" outfits are available. Among those most used are the Sanborn, the Roth, and the Collins modifications of the original "portable" apparatus of Benedict; the Krogh; and McKesson's Metabolor. With all of these, the nose of the patient is closed with a clip, and he breathes through a suitable mouth piece and breathing tubes into an air chamber sealed with a water jacket and containing an excess of O. The expired air is carried through a jar of soda lime inside the chamber to remove CO<sub>2</sub>. The circulation of air may depend upon the patient's own efforts, controlled by flutter valves, or upon a motor blower. By means of a kymograph variations in the volume of air in the apparatus are recorded on special graph paper, from which the diminution in volume (O consumed) can be read directly. This is corrected for temperature, changes in temperature if any, barometric pressure and moisture (the air is 80% saturated), and converted into energy equivalents (calories per hour) by means of tables which are furnished with the apparatus, together with detailed instructions for its operation.

Accurate results depend upon scrupulous attention to details, of which the following are especially important. The test must be carried out in the morning with the patient under "basal" conditions. He must fast for 14 hours before the test. In the morning, before the test, he should not smoke, drink coffee or tea, or make unnecessary physical exertion. A glass of water may be allowed, as this is less disturbing than discomfort from thirst. As a rule the patient may come to the laboratory for the test if he rests at least half an hour before the test. The room must not be cold or excessively hot. The procedure should be explained in detail. Apprehension, restlessness, or discomfort of any sort increases O consumption and leads to gross errors. Complete cooperation of the patient is indispensable. Practically all errors tend to make the result too high. Two periods of six to ten minutes should be tried. If the results are discordant, the lower figure is usually more nearly correct. A third period may be tried, but as a rule

it is better to dismiss the patient and repeat the test the following day. This should always be done if the result is pathologically high, if it is higher than was expected, or if the test appears to be unsatisfactory in any way.

Errors inherent in the use of the apparatus are unusual if reasonable care is taken to avoid them. The most common are: leakage of air, especially around the mouth piece or nose clip, rarely through a perforated ear drum: obstruction to free circulation of air (due to water in the breathing tubes, sticking valves, or caked soda lime); and incomplete absorption of CO<sub>2</sub> because of too protracted use of the soda lime. The latter may give too low a result, but usually causes dyspnoea and irregular breathing. Test occasionally by forcing air out through the O intake tube into a clear solution of barium hydroxide. If clouding occurs, renew the soda lime.

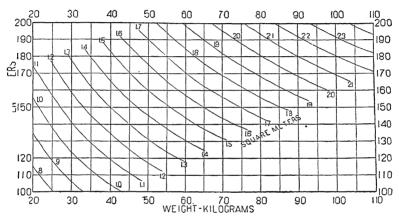


FIG. 186.—Chart for the estimation of the surface area of the body from the height and weight. Plotted from the DuBois formula. (From E. F. DuBois, Basal Metabolism in Health and Disease. Courtesy of Lea and Febiger.)

Significance.—The basal metabolism in normal individuals varies with their age, sex, and size. It varies more nearly with the surface area than with either height or weight. The area may be calculated, from the height and weight, by the DuBois formula:

Area = Height (cm.)
$$^{0.725}$$
 × Weight (kilos) $^{0.425}$  × 71.84

or read by interpolation from the chart.

The basal metabolism is usually expressed as the number of calories produced per Sq. M. of body surface per hour. It is greatest in early childhood and gradually diminishes with age. It is from 7% to 10% lower in women than in men. There is as yet no universally accepted normal standard. The three standards most extensively used are those of Aub and DuBois, based on surface area: of Harris-Benedict, based on height and weight; and of Dreyer, based on weight. We give the table of Aub and DuBois because this is in most general use, and is more dependable for subjects of unusual build. Their figures, which were based on tests in a calorimeter chamber

lasting an hour or more, are about 6% higher than those obtained by most workers by measuring O consumption over 6 to 10 minute periods during which more complete relaxation may be maintained. For children the standards of Benedict and Talbot may be used. The basal metabolic rate is usually expressed as the percentage by which the observed figure deviates from the normal average. The standard with which it is compared should always be recorded. Figures from -10% to +10% (and usually to +15%) are within the limits of the normal variation.

NORMAL STANDARDS OF AUB AND DUBOIS In Calories per Square Meter of Body Surface per Hour

Age	Males	Females
14-16	46.0	43.0
16-18	43.0	40.0
18-20	41.0	38.0
20-30	39.5	37.0
30-40	39.5	36.5
40–50	38.5	36.0
50–60	37.5	35.0
60–70	36.5	34.0
70–80	35.5	33.0

BENEDICT-TALBOT STANDARDS FOR CHILDREN In Calories per Hour from Body Weight

Weight, kg.	Boys, cal.	Girls, cal.	Weight, kg.	Boys, cal.	Girls, cal.
3 4	6.3 8.8	6.3 9.2	2I 22	36.9	34.6
	11.3	11.9	23	37·9 39·2	35.6 36.7
5 6	13.8	14.6	24	40.2	37 - 5
7 8	16.3	16.9	25	41.3	38.8
8	18.5	19.2	26	42.5	39.6
9	20.6	20.8	27	43.5	<b>40</b> .6
10	22.7	22.5	28	44.6	41.7
II	24.6	24.2	29	45 · 4	42.5
I 2	26.0	25.4	30	46.5	43 - 5
13	27.5	26.7	31	47.5	44.6
14	29.0	27.7	32	48.3	45 · 4
15	30.2	28.8	33	49.2	46.4*
16	31.5	29.6	34	50.0	47 · 3*
17	32.5	30.6	35	50.8	48.3*
18	33 · 5	3 <sup>1</sup> .7	36	51.7	49.2*
19	34.6	32.5	37	52.3	50.1*
20	35.8	33 - 5	38	53 . I	50.7

<sup>\*</sup> These figures obtained by interpolation.

Alterations in Disease.—The B.M.R. is controlled primarily by the thyroid through its secretion of thyroxin. In severe cretinism and in myxoedema and after total thyroidectomy the B.M.R. falls to a fixed level about 40% below the normal. It can be elevated with relative precision by intravenous injections of thyroxin, rising after a latent period of 12 hours 3% for each 1.0 mg. administered. In cases of thyroid intoxication, whether due to exophthalmic goiter or toxic adenoma, the B.M.R. is elevated (+25% to +100%), the degree of increase being proportional to the acuteness and severity of the disease. Repeated estimations of the B.M.R. afford the best single means of determining the results of treatment and the optimum time for operation.

Fever from any cause results in an elevation of the basal metabolic rate of about 7.2% for each degree F. (13% for r°C.). Elevation of the B.M.R. is also seen in leukacmia in the active stages, in many cases of polycythaemia, in some cases of pernicious anaemia, in some cases of pituitary disease with hyperplasia of the anterior lobe, and after the administration of certain drugs, particularly epinephrin, ephedrin, dinitrophenol and (in less degree) caffeine.

Mild degrees of hypothyroidism characterized by asthenia, easy fatigue, sensitiveness to cold, bradycardia and constipation, with a B.M.R. of -15% to 30%, are common. They are usually benefited by the administration of thyroid in amount sufficient to raise the rate moderately toward the normal. A moderate reduction is common in malnutrition and cachexia from any cause. A reduction is seen also in a few cases of obesity, in hypopituitary conditions (Froelich's syndrome, Simmond's disease), in some cases of Addison's disease, in "nephrosis," and in some psychoses accompanied by markedly apathetic depressed states.

A few apparently healthy individuals are met with who show a B.M.R. substantially below the normal figure. If their rate is raised to the usual normal by treatment or by disease, they may show signs of thyroid intoxication. They are peculiar in that their metabolism appears to be set at an unusually low level.

Reed's formula.—In individuals with no abnormality of the cardiovascular system the resting pulse rate is usually proportional to the B.M.R. Reed has suggested the following formula for calculating the B.M.R. from the pulse rate and pulse pressure.

B.M.R. = 0.685 (Pulse rate + 0.9 Pulse pressure) - 71.5 Example: Pulse rate 100. Pulse pressure 70 (150 - 80).

B.M.R. =  $0.685(100 + 0.9 \times 70) - 71.5$ = +30.8%

Reed reported that the error was less than 10% in 60% of the cases and less than 20% in 90% of the cases.

#### PREGNANCY TEST

Aschheim-Zondek test.—This is not a direct test for pregnancy, but for the anterior pituitary-like gonadotropic hormone (prolan) which is present in the urine in large amounts during pregnancy. It is so arranged quantitatively that the (usually) small amounts present in other conditions are not revealed.

Procedure.—Five infantile mice from 3 to 4 weeks old and weighing 6 to 8 Gm. are each given subcutaneous injections of 0.5 cc. of urine twice daily for three consecutive days. If the urine is toxic for the mice, this difficulty can sometimes be remedied by extraction with ether. About 96 hours after the first injection the mice are killed and the ovaries are examined with a hand lens for:

Reaction 1: Enlarged follicles.

Reaction 2: Haemorrhages into the follicles (Blutpunkte).

Reaction 3: Corpora lutea.

The occurrence of Reaction r only is suggestive, and the test should be repeated. Reaction 2 or 3 is definitely positive. Occasionally positive reactions may be evident after 60 hours. They reported 99.5% negative reactions in 1075 non-pregnant individuals, and 98% positive reactions in 925 cases of pregnancy. Later work by many different investigators fully confirms their results and indicates that the error does not exceed 2%.

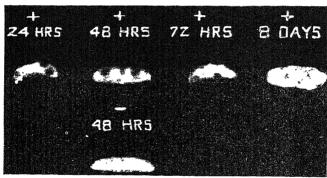


Fig. 187.—Gross photograph. Rabbit ovaries. Natural size. Gross appearance of positive test of one, two, three and eight days' duration and of negative test. (After A. M. Young, from Journal of Laboratory and Clinical Medicine.)

Friedman Test.—Friedman (1929) recommended the use of a single mature female rabbit in place of the mice. The animals were isolated in individual cages for three weeks, and then given 24 cc. of urine intravenously in divided doses. About 48 hours after the first injection the animal is killed and the ovaries examined. The presence of haemorrhagic follicles constitutes a positive reaction. This method with minor modifications has been used extensively in the United States with an error not exceeding 5%.

We recommend the following procedure described by Young (1934), in order to reduce the errors to a minimum. (1) Select healthy female rabbits weighing 2 to 4 kilos. (2) Isolate in individual cages. (3) Under morphine anaesthesia (1/2 grain intravenously) open the abdomen and inspect the ovaries, selecting animals with well developed follicles but without corpora haemorrhagica or corpora lutea. (4) Secure a specimen of urine voided in the morning with a Sp. Gr. of at least 1.015. If necessary restrict fluids the previous day to secure the needed concentration. Filter clear, warm to body temperature, and slowly inject 15 cc. intravenously. Repeat the injection

after 4 hours. (5) After 48 hours again inspect the ovaries. If they appear positive on gross inspection, remove one ovary and examine microscopically a frozen section. The presence of lutein tissue and some haemorrhage in one or more follicles constitutes a positive reaction. (6) If the ovaries appear normal, a tentative negative report may be made. Close the abdomen and inject as a control 15 cc. of known positive urine (or a suitable concentrate). (7) After 48 hours again inspect the ovaries. If corpora haemorrhagica are present, indicating that the animal was capable of a normal response, the reaction (in (6)) may be regarded as definitely negative.



FIG. 188.—Photomicrograph. High power. Rabbit ovary. Corpus luteum of forty-eight hours duration showing proliferation of capillaries. (After A. M. Young, from Journal of Laboratory and Clinical Medicine.)

If it is not possible to prepare sections, the ovaries should be inspected again on the fifth or sixth day. If positive, corpora lutea will then be unmistakable in gross. If the urine is toxic for the rabbits, the hormone may be concentrated by adding to 60 cc. of urine, 5 volumes of 95% alcohol. Centrifugalize in large tubes, decant, and dissolve the precipitate in a few cc. of distilled water. Extract for 15 minutes with 3 volumes of ether; aerate to remove the ether, and inject the aqueous solution (volume of about 10 cc.) slowly into the vein.

If the preliminary inspection of the ovaries is carried out, the initial period of isolation of the rabbits may be shortened or omitted, although Young found 20% of her stock animals unsuitable. Animals usually may be used a second time if they are isolated at least a month after the first test.

By the use of these precautions errors due to individual variations in the test animals are practically eliminated. In her series Young reported one false negative in 96 cases of pregnancy, and 4 false positives in 135 non-pregnant individuals, 3 of whom had teratomatous tumors of the ovary or testis. The test, therefore, compares favorably in accuracy with the original Aschheim-Zondek method.



Fig. 189.—Photomicrograph. Low power. Rabbit ovary. Positive test showing follicles of twenty-four hours' and of eight days' duration. (Corpus haemorrhagicum and corpus luteum.) (After A. M. Young, from Journal of Laboratory and Clinical Medicine.)

The test usually becomes positive within a few days after the first missed menstrual period. Positive tests have been reported 10 to 16 days after conception before a period has been missed. It becomes negative within a week after delivery unless membranes are retained. It is positive in ectopic pregnancy as long as intact chorionic villi are present. Disappearance of a positive reaction usually means death of the foetus.

Positive reactions are regularly found in cases of hydatidiform mole, chorionepithelioma and teratomata containing chorionic epithelium in both men and women. The reaction becomes negative quickly after removal of the tumor. The persistence or reappearance of a positive reaction indicates incomplete removal or recurrence.

## CHAPTER XLII

# FOOD DEFICIENCY DISEASES

THE avitaminoses are diseases which result from a deficiency of one or more of the vitamins. The relationship of a deficient, ill-balanced diet to certain of these diseases has been recognized for two centuries, but the fact that disease may result from the lack of minute amounts of certain specific substances in a diet adequate in caloric value, in protein content, and even in mineral salts, has only been revealed by the work of the last 30 years.

The vitamins are organic chemical compounds, each of which is entirely different from the others. Each has a specific function which no other will perform. Normal growth and development depend upon an adequate supply of all. Certain functions require the activity of several vitamins—for instance the development of the teeth and peridental tissues seems to be dependent upon A, C, and D. Reproduction may be affected by a lack of either A, B, D, or E, although the type of sterility produced by a deficiency in each differs. Maintenance of the resistance to infection has been attributed to various vitamins. The chemical structure of the vitamins is very complex, and it is probable that some of those recognized at the present time will be split further into simpler chemical substances with more limited physiological activities. Vitamin E, for example, may be composed of 2 substances, one influencing reproduction and the other lactation; and even the comparatively well known antiscorbutic vitamin is now believed by Szent-Györgyi to consist of 2 fractions. Animals of different species may not be affected in exactly the same way by a lack of a certain vitamin, and it is not always possible to ascribe to man the effects produced in the experimental animal. We are almost entirely ignorant of the precise physiological activities of the vitamins. B<sub>1</sub>, B<sub>2</sub>, and C seem to be associated with various oxidation processes. A relationship between the activities of certain vitamins and those of the endocrine glands is suggested, but not substantiated. We have attempted to summarize the present knowledge of the more important properties and activities of the different vitamins in the Table.

The precise quantity of each of the vitamins required to maintain health is not known and probably varies with different individuals even under the same conditions. The figures given in the table are those of Salter, which are considered by some authorities to be too low. The requirements for one or more of the vitamins may be increased temporarily by various factors such as rapid growth, physical exercise, pregnancy, infections, etc. Furthermore, a deficiency may result not only from an inadequate intake but also from faulty absorption (which may be selective) or storage. Experimental work on animals suggests that the need for vitamin E occurs only during a

Important Attributes of the Vitamins

	Remarks		Barly diagnosis possible by testing rate of regeneration of the regene	The original B complex contrains at least 5 or 6 different active fractions, as determined in rats and pigeous. The significance of the other fractions for man is not known. Writerstein and Schon suggest that Bi and Bi prevent beri-ber and Bs and B, and B, and B, and B, prevent beri-ber and Bs and B, pellagra.	Lactoflavin is a fluor- escent photosensitive yellow pigment which readily undergoes re- versible oxidation-re- duction reactions and plays an important
IMPORTANT ALLKIBOLES OF THE VITAMINS	Approximate daily requirement.† Best sources for treatment.		Cartene 0.3 mg. (Salter). Fish Wher oil especially halibut. Richest source oil (Sarendepi). gigas). Green leaves.	Witamin Bi o.5 mg. (Salter). Maintenance I. Jo mg. Cura- tive 10 mg. (Vorhaus et al.) Vorhaus et al.) Oli shin gs. Wheat embryo. Peast. Legumes.	Not known. Yeast. Wheat germ. Liver. Egg—flavin. Rish—Bs.
	Avitaminosis	Experimental animal			In rats and dogs a deficiency causes abnormalities of skin and mucous membranes resembling pellagra (dermatitis, black
	Avitan	Human	Xecrophthalmia. Night blindness. Diminished resistance to in- sistance to in- fection. Xeresis of kerin. Steril- ity from meta- plasia. Dwarf- ism?	Beriberi. (May be a factor in be a factor in be a factor in meuritis). Castrointestinal atony. anorexia. Sterility.	One factor in the production of pellagra. Dermatitis. Nutritional cataract. Impaired lactation.
	Physiological effects.*		Bssential action— maintains normal col- ummar and cuboidal optichium, especially of respiratory, gastro- intestinal and gantio- urinay tracts, eyes and I ac hr yn ma I ghands. Also skin and possibly nervous system. Promotes re- sistance to penetra- tion of pathogenic organisms. Influences reproduction and lac- tation.	Essential action—prevents degeneration of peripheral nerves. Maintains tone of east-order and stimulates appetite. Infleries reproduction and lactation maintains octrons cycle. More required at puberty. Reported to increase glucose tolerance and lessen accumulation of lactica acid.	Both factors required for normal growth. Be probably the anti- pellagra factor (Gy- órgyi).
	Units. International standard.		Viramin A ac- tivity of 0.67 (0.6 micro- grams) of grants acrotene (crys- talline).	Vitamin B1 accivity of ton mg of the International Standard at 8 or p tion in product of rive pol 18 hings. (This contain a but but small later use crystalline B1.	None
	Chemical and	physical properties	Provitamin. B-carotene (4iso ac and y-carotene and crypto-acathene and crypto-acathin). One-sep-onitable. Soluble in fat solvents. Beatly destroyed by oxidation of he raw is estable. Resists hear, 250°C. Absent from Trancid fats. Adsorbed by Ca(OH)s. CosHab (§ Carotene CosHab) (Karrar, 1931).	Soluble in water, glycenein and alcohol. Insoluble in ether, according to the soluble in edge. Destroyed by heat, 130°C. Adsorbed by Fuller's earth. Stable in acid, not in alkaline solution. Isolated as crystals. CuHisON S. (Windsaus, 1932). CuHison N.O.S. (Williams, 1934).	Soluble in water and dilute alcohol. Insoluble in fat solvents. Heat stable. Stable to acids, moderately to alkalis. A complex of (1) Lacto-
			A	· <u>K</u>	Br (G) (P.P. factor)

part in oxidation reactions of all cells. Reducing agents or light change it to an inactive (letroc-) form. Shaking with air oxidizes it into original form. Ir- radiation of a diet renders it dermattis- producing, possibly because lactoflavn is blaached.	Parenteral administration of sodium salt of evitamic and to evitamic and buffered orange juice can be carried out when absorption is deficient. Stored to some extent in all tis sues, even in crystal-line lens. Most abundant in adreand cortex, pain, pancreas, anterior lobe and especially pars intermedially pars inter	Exists in several differ- ent chemical forms. Vitamin D in irradi- ated ergostered differs from D in cod liver oil, is most effective in ratis and relatively ineffective in pigeons. Contains a phenan- thren nucleus like the cestrogenic hor- mones and the car- cinogenic hydrocar- bons. Vitamin D also cestrogenic. Cod liver oestrogenic. Cod liver oil can be given par- enterally. Absorbed through skin. Hy-
	Cevitamic acid 3 mg. (Salter) 30 mg. or 30 cc. 60 crange juice (Harris). Synthetic cevita thetic cevita mic acid. Cit- rous f ruits. Raw tomatoss. Lettuce, cab- bage. Germin- atting peas and beans.	Viosterol o.o.s mg. (Salter). Calciferol. Fish fiver oils. Milk fiver oils. Milk and eggs if an- ands are irradi- ated. Various other foods if irradiated.
tongue). Cata- ract.	Guinea pigs de- velop typical scurry after 3 weeks with exten- sive haemor ringges, especially in joints, and de- fective bone and tooth formation tooth	Rickets in rate. ocoon mg ordless ocoon mg ordless ocon curl ordless to the rate of the ra
	Scurvy	Rickets. (1) Low phos- phorus type. clum type with tetany. Osteo- malacia. De- layed dentition. Dental carties.
	Basential action— mantains the inter- cellular cement sub- stance of comertive tissues. Maintains integrity of capillary walls. Basenthal for development of bones and teeth. Utlized in in large amounts in in large amounts in cette uffections. In- active the some vir- uses and toxins. In- thereces ha e mo there was a cativate some vir- uses and toxins. In- thereces ha e mo toxing a control thereces ha e mo ovygen carrier in cellular metabolism.	Regulates calcium and phosphorus metabolinism (bothes and teeth) in conjunction with the quantity and ratio of Ca and Pin the diet, and with parathormone. Promotes absorption (or lessens re-exerction) of calcium from gastrontestinal tract. Some believe that it acts by stimulating parathyroids (?)
	Vitamin Cactivity of 0.05 mg. acid. Lascorbic	I mg. of Inter- national Stand- ard solution of  grandated er- grandated
flavin, CrHesN.O., and probably (2) Bs (Györgyi); structure unknown.	Cevitamic (l-ascorbio) acid. Structural formula known. Can be synthesized. White crystals in rectangular plates soluble in water, dilute alcohol and acids. Insoluble in fat solvents. Very easily oxidized (reversible reaction). Destroyed by prolonged heating in presence of 0, not by moderate heating in vacuo. Cal40, e(ox and Hurst, 1932).	Provitamin—ergos- terol and possably other sterols. Vita- min—calciterol or viosterol, prepared by irradiation of ergos- terol. Porms crystals soluble in fat solvents. Moderately resistant to heat and oxidation. Destroyed by over- radiation. Found in unsaponifable frac- tion of animal and unsaponifable frac- tion of animal and vergetable fats. At least 5 isomers of ir- radiated ergosterol occur of which only
:	. 0	Ф

IMPORTANT ATTRIBUTES OF THE VITAMINS (Continued)

	Remarks		pervitaminosis in rat, dogs, etc, causes abnormal deposition of calcium in various tissues and may be fatal. Huge dosse cause intoxication in man also.	D. 3227 8 37	
	Approximate daily requirement.† Best sources for treatment.			Not known. Wheat germ oil. Whole wheat (creal. and bread. Cotton- seed oil. Let- tree, yellow corn, egg yolk, beef liver, etc.	
rvs (commuted)	Avitaminosis	Experimental animal		Rats grow normally but females exhibit a characteristic sterility. Poetuses are absorbed (shown by a gradual loss in weight). Males show a permanent degeneration of the germinative critical acrolls and atrophy of prostate and seminal resicles.	
Continued	Avita	Human		Habitual abortion? Successful treatment of some cases h as be en cleamed. Need for intramin man not yet proved.	
	Physiological effects.*			Maintains fertility in male and female rats. Required for lacta- ferent substance).	h and mandaction
and the second s	Units. International standard.			None	for normal ground
TO STATE OF THE PARTY OF THE PA	Chemical and physical properties calcifered is antirachitic. Ca, H. (a) Wind-aus, 1932).		calciferol is antirachi- tic. Calluo (Wind- aus, 1932).	Soluble in oils or fats. Thermostable, Unstable to oxidation. Partially destroyed by ultravoice light. My ultravoice light, hydrogenation caponification, inactivated in rancif fats. Almost identical with cholesteroi (SaHaO). (Evans, 1935).	* All vitamins are essential for normal mounth and manadactical
				ല	* A1

\* All vitamins are essential for normal growth and reproduction. Only those attributes have been given, which now seem fairly well established. See text. oce tax.

Precise figures can not be given. The requirements are variable, and are influenced by diet, infections or other diseases, etc. Some believe that Salter's figures are too low.

critical period in foetal life. It is quite possible that deficiencies in childhood may have a direct bearing upon health in adult life.

A serious deficiency in any one of these vitamins leads to a well marked and distinctive symptom-complex which is readily recognized. Under natural conditions, however, a diet notably deficient in one vitamin is likely to be inadequate in its content of one or more of the other vitamins and of other food substances, and the clinical symptoms resulting from such a diet may be complex and difficult to interpret. In fact, the existence of *pure* deficiency diseases has been questioned. Each condition shows some features of deficiency in other vitamins. Outspoken cases of most of the deficiency diseases are rarely encountered at present in civilized countries, but vague manifestations of ill-health due to inadequate quantities of one or more of the vitamins are more common than is generally realized. It is difficult or impossible to recognize these latent cases with certainty on clinical grounds alone, and there is great need for precise laboratory tests for specific vitamin deficiencies such as are available for the antiscorbutic vitamin.

The comparative values of different foods as sources of vitamins  $A,\,B_1,\,B_2,\,$  and  $C,\,$  based on the work of many investigators, are indicated in the following table. The values as set forth are not to be taken too literally. As pointed out by McCollum there is no adequate procedure for estimating precisely the quantity of any vitamin. Unless this is borne in mind the table may be misleading.

#### Values:

- + indicates that the food contains the vitamin but is not a good source.
  - · indicates that the food is a good source.
  - indicates that the food is an excellent source.
- indicates that the food does not contain the vitamin.
- $\pm$  indicates that it is doubtful if the food contains the vitamin.
- ? indicates a lack of experimental evidence.

### VITAMINS IN FOOD

Classes of foodstuffs	neuritic pell prev	or G C anti- agra- scorbutic entive factor
Meat, fish, etc.:		
Lean meat (beef, mutton, etc.)	- to +	- to +
Lean pork		
Poultry		
Liver		
Kidneys		
Heart		

# VITAMINS IN FOOD (Continued)

Classes of foodstuffs		B <sub>1</sub> anti- neuritic factor	B <sub>2</sub> or G pellagra- preventive factor	C anti- scorbutic factor
Brain Sweetbreads Fish, muscle, lean Fish, roe Oysters Clams Dairy products:	- to +	- to + - to +		
Milk, cow's whole, raw				+ to +
Milk, boiled whole.  Milk, condensed. Cream. Cheese, whole milk. Cheese, skim milk. Eggs, fresh whole. Fats and oils:			- to +	to + to +
Butter. Cod liver oil. Mutton and beef fat or suet. Lard. Cottonseed oil. Olive oil. Peanut oil.	+++ +++ + ± ±			
Margarine prepared from animal fat  Margarine from vegetable fats or lard  Nut butters	- to ++ - - to +			
Vegetables: Leaves, stalks, flowers: Asparagus Cabbage, fresh, raw Cabbage, fresh, cooked Cauliflower Celery Chard	- to +	+++ ++ ++ + +	+++ ++ ++ ++ + ;	
Lettuce. Rhubarb. Spinach. Squash, Hubbard. String beans.		; +	; ++ ;	±
Roots, bulbs, tubers: Beet root. Carrots, fresh raw Onions, raw			+ + + + +	- to +
Onions, boiled. Irish potatoes (cooked with skin). Sweet potatoes, cooked. Rutabaga.	- to + +++		+ + + + + + +	- to + + to ++ + to ++
Cereals: Barley, oats, rye, whole grain Wheat, rice, whole grain	- to +		++ +	

# VITAMINS IN FOOD (Continued)

Classes of foodstuffs		B <sub>1</sub> anti- neuritic factor	B <sub>2</sub> or G pellagra- preventive factor	C anti- scorbutic factor
White wheaten flour, pure corn-flour, polished rice, etc. Germinated cereals. Yellow maize. Legumes: Beans, kidney. Beans, Navy. Beans, soy. Peanuts. Peas, fresh Germinated legumes	± +	- to + ++ +++		++
Pruits: Apples. Bananas. Cantaloupe. Bggplant Grapefruit. Grape juice. Lemon juice, fresh. Lime juice, fresh. Orange juice, fresh. Peaches, yellow Prunes. Raisins. Raspberries. Strawberries. Strawberries. Tomatoes, fresh. Tomatoes, canned Watermelon Nuts: Almonds. Cocoanut. Pecans	++++++		++ + ? ++	++ +++ + +++ ++ ++ ++ ++ ++ ++ +++ +++
Pecans Walnuts (English) Sugar and starches: Honey. Sugar Miscellaneous: Bread, white (water) Bread, white (milk) Yeast Meat extract Beer Alfalfa and clover	+++			— to +

# NIGHT BLINDNESS AND XEROPHTHALMIA

Night Blindness and Xerophthalmia are the result of a marked deficiency in vitamin A. They are common in certain parts of the tropics in which the diet is apt to be deficient in this so-called "anti-ophthalmic" vitamin, especially in regions which are subject to famines.

Night blindness (nyctalopia) was noted often among the crews of sailing ships, especially when they were becalmed in tropical waters. For a time it was believed that the heat and sunlight of the tropics were important factors, but later work has shown that a deficiency in vitamin A is responsible. This inability to adapt the vision promptly to a faint illumination is due to a disturbance of the metabolism of the visual purple in the rods of the retina. Bright light leads to a bleaching of the purple which normally is regenerated quickly. Vitamin A deficiency causes a delay or failure of this regeneration. The suggestion has been made that this is a reversible reaction, in which the vitamin A is utilized in the synthesis of the purple and liberated when the purple is bleached.

Night blindness may be the first clinical evidence of a deficiency in vitamin A. Jeans and Zentmire have devised a simple method of testing light sensitivity by which early latent avitaminosis may be recognized. In 20% of the children examined a definite reduction in sensitivity was found which cleared up promptly when vitamin A was added to the diet.

Xerophthalmia may occur when the deficiency in vitamin A is marked. The first manifestations are dryness of the conjunctivae and irregular areas of xerosis here or on the cornea. Dry triangular areas adjacent to the cornea, covered with a foam-like crust (Bitôt's spots) are particularly characteristic. Later the process spreads over the cornea and the remaining conjunctiva, so that the whole surface of the eye becomes dry, wrinkled, opaque and yellowish, like skin. Ulcers appear on the cornea, and secondary infection occurs which often leads to sloughing and complete blindness. Very little pain is felt. There is often a brownish pigmentation of the conjunctivae in dark-skinned races. The dryness of the eye is due in part to a lack of secretion of the lachrymal glands. A similar change in the salivary glands leads to dryness of the mouth and throat and may cause aphonia.

There is a characteristic dermatitis which, according to Loewenthal (1933), is not uncommon and may precede even the night blindness. Small, red, dry, keratinized papules resembling goose flesh appear and are most numerous on the extensor surfaces.

Pathology.—The primary lesion consists of a metaplasia of the ectodermal structures with a transformation of the normal epithelium into stratified keratinized epithelium. This process involves the eye, the lachrymal glands, the salivary glands, the mouth and throat, parts of the respiratory tract, (larynx, trachea, bronchi), the gastrointestinal tract, BERIBERI 797

the genitourinary tract, and even the skin, which may become dry, scaly, shriveled and pigmented.

This change in the epithelium is associated with a marked lowering of the local resistance to the penetration of microorganisms into the tissues, and the patients become peculiarly susceptible to respiratory, urinary tract and other infections. It is still controversial whether there is in addition a lowering of the general defensive forces of the body.

In experimental animals vitamin A deficiency often leads to the formation of renal calculi and sometimes deposition of calcium in the renal tubules. There is, however, no direct evidence that this is concerned in the formation of urinary calculi in man. Sterility also occurs in experimental animals and is due to persistence of cornified vaginal cells which interfere with implantation. In young puppies vitamin A deficiency causes degenerations of the spinal cord characterized by demyelinization of the nerve fibres. It has been shown also that vitamin A will neutralize the neurotoxin of ergot. These facts have suggested to some that a deficiency in A plays a part in ergotism, lathyrism, pellagra, and even in pernicious anaemia and disseminated sclerosis.

Treatment.—Fish liver oils, especially halibut, are the most potent sources of vitamin A for treatment. It is also present in butter, milk, eggs and vegetables containing green or yellow pigment. Spence showed that 15 cc. of cod liver oil daily would relieve night blindness in from 3 to 5 days and clear up Bitôt's spots within 5 or 6 weeks.

### Beriberi

Beriberi is an important disease in the Orient and in communities, especially in the tropics, where rice is used as a staple article in the diet. The characteristic lesion is a degeneration of the peripheral nerves, similar to that due to alcohol, arsenic, or diphtheria toxin, except that there is practically always a marked involvement of the vagus nerve and of the heart muscle. It is generally conceded that the disease is due primarily to a deficiency of the Br component of the B vitamin complex in the diet.

Eijkman (1895) showed that a diet of milled rice caused a peripheral neuritis in fowls, and that this disease could be prevented or cured by adding to this diet the rice "polishings" which had been removed in the process of milling. These observations have been extended to man by Eijkman and others, and the fact that beriberi can be induced by a diet consisting mainly of milled rice or other milled grains has been amply demonstrated.

Fraser and Stanton have shown that the antineuritic vitamin and much of the protein is contained in the pericarp and aleurone layers and in the embryo of the rice. These are more or less completely removed during the process of milling. It has been found that, although the vitamin itself does not contain phosphorus, the content of vitamin remaining in the grain after milling parallels roughly that of phosphorus pentoxide. These investigators believe that the use of rice containing less than 0.4% of  $P_2O_5$ , as a staple article of the diet may cause beriberi.

Clinical picture.—The period of development of symptoms is three months or more after the beginning of the inadequate diet and varies

markedly with different individuals. There are two clinical types of beriberi: (1) dry or paraplegic and (2) wet or dropsical, but this distinction is an artificial one, for many of the cases present features of both types during the course of the disease. In the former type the patient complains first of muscular weakness and numbness, especially in the legs. and of a sense of fullness and tenderness in the epigastrium. Cardiac palpitation and dyspnoea follow the least exertion. The patellar reflex is at first hyperactive, but disappears within a week or two. Hyperaesthesia of the calf muscles and blunting of sensation in the hands and feet become noticeable. There is frequently oedema over the tibiae and patchy areas of oedema may occur anywhere in the body. Later foot drop. wrist drop, general muscular atrophy and paralysis may appear. Paraplegic cases frequently show the characteristic tripod gait. Leaning forward on a cane with the legs apart, they resemble a tripod. With each step the feet are lifted high and swung outwards to avoid scraping the toes. This type may show a positive reaction to the so-called "jongkok" test: inability to rise from a squatting position with the hands held over the head. In severe cases the paralysis may extend to the diaphragm and intercostal muscles.

The wet type of beriberi is characterized by a marked dilation of the heart, particularly of the right side, and general oedema and effusions into the serous cavities. The blood pressure is always low, particularly the diastolic pressure, with a corresponding increase in the pulse pressure. Tachycardia occurs after the slightest exertion. The normal rhythm of the heart is replaced by the equal spacing of embryocardia, and systolic murmurs, due to incompetency of the valves, especially the tricuspid, are audible. Sudden death from failure of the right side of the heart is frequent in these cases and may occur in any type of beriberi. This is commonly called "shôshin" and may occur within a few hours after the onset of symptoms. Vomiting is uncommon, but when it occurs, the prognosis is bad.

Atypical cases are frequently encountered. In the so-called "rudimentary form" there may be merely weakness of the legs with some anaesthesia, variable reflexes or slight oedema.

Vorhaus et al. (1935) ascribe to vitamin B<sub>1</sub> deficiency a syndrome characterized by hypotonicity of the gastrointestinal tract, anorexia, constipation and vague muscular pains, and believe that states of slight deficiency are much more common than has been believed.

The adrenalin test suggested by Aalsmeer and Richter (1931) has been used for diagnosis and to determine the result of treatment. The test depends upon the fact that an injection of adrenalin produces in beriberics a marked fall in diastolic pressure

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(almost to zero). In a later report (1935) Aalsmeer states that occasionally the reaction may fail owing to decompensation of the left heart, but a repetition of the test after a preliminary dose of cardiazol will elicit the characteristic response. The reaction may persist after the symptoms are relieved, and he regards a positive test as an indication that the disease is still active.

The pathological lesions consist of degeneration of various peripheral nerves and of the vagus, with associated changes in the cells of their nuclear centers, and a secondary muscular atrophy. All of the tissues, including the heart muscle, are oedematous, and the serous cavities are often filled with fluid.

The cause of this water retention in beriberi is not well understood. The patchy areas of oedema have been ascribed to involvement of the local vasomotor nerves, and the general anasarca to failure of the heart muscle. This is believed by some to be due to involvement of the cardiac branches of the vagus, while others ascribe it to mechanical interference with the contractility of the heart muscle by the oedema. Others suggest that the oedema is due to an associated protein deficiency such as occurs in nutritional oedema. Determinations of the plasma proteins, however, have shown no abnormalities. Although the basal metabolism has been found to be low in cases with oedema, as in other types of oedema, it rises to normal when the water balance is reestablished.

McCarrison has noted hypertrophy of the adrenals with an increase in the adrenalin content of the cortex and atrophy of the other endocrine glands (except the pituitary) and suggests that some of the clinical manifestations may be due to endocrine dysfunction.

Etiology.—It is generally conceded that beriberi is caused primarily by a diet deficient in the antineuritic vitamin  $[B_1]$ . However, peculiarities in the disease itself and in its distribution suggest that other factors are also concerned. Some authorities believe that the principal factor is a (hypothetical) toxin present in damaged rice, and Chick (1933) suggests that vitamin  $B_1$  acts as a detoxifying agent. Beriberi would occur, therefore, only in individuals whose diet consists almost exclusively of rice containing this toxic substance and is deficient in  $B_1$ . This may explain the fact that in India where millions subsist on polished rice, the disease occurs only in a few areas. There is, however, no direct evidence of the existence of a toxin.

Minot et al. (1933) have advanced evidence that a deficiency in vitamin  $B_1$  is the cause of *alcoholic polyneuritis*, either from a limited intake of food or a lack of absorption by a damaged gastrointestinal tract. They report cure of the neuritis by the administration of large amounts of B complex orally and parenterally, even in patients who continued to receive the quantity of alcohol to which they were accustomed.

Excessive amounts of carbohydrate in the diet increase the need for  $B_1$  and may result in the development of symptoms of beriberi.

Beriberi and pellagra have been observed in the same individual, but this is rare. Cowgill has suggested as an explanation for this fact that in the Orient where the diet consists largely of polished rice and fish, the fish supplies sufficient  $B_2$  to prevent pellagra. In regions where pellagra is common the diet consists largely of maize, which is poor in  $B_2$  but contains adequate  $B_1$ .

Treatment.—When placed on an adequate diet rich in vitamin B<sub>1</sub>, beriberics usually improve at once, although it may take weeks or months to repair the damage to the peripheral nerves. It is considered advisable by some to eliminate rice entirely from the diet. Yeast or dried yeast concentrates and extracts of rice polishings (tiki-tiki, practically free from B<sub>2</sub>) are the best sources of the vitamin. Milk is a good source, even when pasteurized, for the vitamin resists heat well (it is destroyed at 130°C.). Eggs, liver, kidney, and most vegetables are good sources.

Acute pernicious beriberi.—This form is more common in individuals whose diet has become abruptly deficient in the antineuritic vitamin. The involvement of the vagus overshadows the other phenomena, and death may occur within a few hours.

Infantile beriberi.—This type affects infants nursed by beriberic mothers, even those who have only a latent symptomless deficiency. The onset is usually abrupt with restlessness, vomiting, altered voice, tachycardia, oedema, cyanosis, and rigidity. Death may occur within a few hours from acute heart failure. The pathological lesions are similar to those of the adult types, but there is less evidence of degeneration in the peripheral nerves. Occasionally the disease appears in a chronic form in which constipation and vomiting are the prominent symptoms.

The mortality in beriberi is nearly 100% in the acute pernicious type, about 50% in the wet type, and may be as low as 2% in the other types.

Ship beriberi.—This is a disease resembling typical beriberi, which was prevalent among the crews of ships on long voyages. It was particularly common on the Scandinavian ships. The main features are oedema of the legs, dyspnoea, cardiac palpitation and occasionally death from dilatation of the heart. The chief difference in the two diseases lies in the absence of any manifestations of peripheral neuritis. Nocht noted sore gums and haemorrhages into the muscles in a few cases and considers that the disease is closely related to scurvy, particularly to the condition known as Rand scurvy.

It is quite probable that ship beriberi and other atypical forms of beriberi may be due to diets which are deficient, not only in the antineuritic vitamin, but also in other vitamins and perhaps in essential minerals.

## PELLAGRA

Pellagra is a chronic, relapsing, wasting disease which is due to a dietary deficiency and which is characterized by gastrointestinal disturbances, degenerative changes in the nervous system, and a peculiar symmetrical cutaneous eruption. It occurs in temperate regions as well as in the tropics. Outbreaks appear in the spring (and often, less severely, in the fall), perhaps as a result of a greater dietary deficiency during the winter. It is not contagious.

Pellagra is largely limited to the poorer classes, especially to those on farms. Town dwellers are relatively free. Outbreaks have occurred among inmates of prisons and

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asylums. It is common among chronic alcoholic vagabonds, and an acute attack may be precipitated by a protracted debauch of several weeks during which the diet is extremely meager in quantity and variety. Pellagra affects both sexes, but in the United States more of the cases are in women. The period of development is from 9 to 12 months according to Sandwith.

Clinical picture.—The acute attack may be preceded by vague digestive discomforts and "neurasthenic" complaints: headache, vertigo with a tendency to fall, generalized pains, depression or changes in disposition. The onset may be marked by stomatitis with desquamation of the lingual epithelium and ulceration, local pain, pyrosis, eructations, nausea and diarrhoea.

The most characteristic feature is the skin eruption. Symmetrical scaly patches of erythema resembling sunburn appear suddenly on the most exposed (or irritated) parts of the skin. The commonest sites are the backs of the hands and wrists, often with a narrow band extending across the palmar surface of the wrist joint; the bridge of the nose; the neck, where it often resembles a collar; and occasionally on the dorsum of the feet, and on the scrotum or female genitalia. Exposure to sunlight aggravates the burning and itching and sometimes seems to precipitate an outbreak. The patches are erythematous in Caucasians, blackish or purplish in negroes, and sepia in the olive skinned races. In severe cases there may be vesiculation and the development of a moist eczematous condition. Petechiae on the affected parts are common. The eruption usually subsides after about a fortnight.

In rare cases the disease runs an acute course and death may occur within a few weeks. Usually symptoms abate in two or three months, but recur the next spring in severer form. If not treated this may continue for ten or fifteen years before death ensues. In such chronic cases emaciation becomes extreme. Serious psychoses may develop, characterized by depression, a tendency to suicide, delusions, confusion and general mental deterioration. Combined sclerosis of the spinal cord is common, with ataxia, spasticity, weakness or paralysis and incontinence. There is usually a subacidity or achlorhydria and often an anaemia, usually hypochromic in type.

The prognosis is good, except for the aged, if the patient is treated early, before the central nervous system is involved. It is unfavorable in very acute, stuporous, or advanced cases.

Etiology.—Most experimental evidence supports the view that pellagra is due, at least in large part, to a deficiency of the P.P. (pellagra-preventive) factor discovered by Goldberger and co-workers, a water-soluble thermostable constituent of yeast, now called vitamin  $B_2$  (or G). Early observers ascribed pellagra to some toxic factor present in a diet consisting largely of maize and deficient in meat. There is often a striking correla-

tion between the consumption of maize and the incidence of pellagra in the population. Later others thought pellagra was due to some infectious agent. In 1915 Goldberger induced pellagra in 6 of 11 volunteers by a diet rich in carbohydrate and poor in vegetables and protein. Sixteen other volunteers tried for six months without success to infect themselves in various ways with material from pellagrins. This effect Goldberger at first attributed to inadequate amounts of protein or to the use of proteins of low biological value (deficient in certain essential amino acids). Maize protein is very poor in this respect. Later he discovered the preventive and curvative effect of yeast, both in black tongue in dogs and in pellagra in man.

Although the relation of a dietary deficiency to pellagra seems well established, the exact nature of the deficiency is in dispute. The diet used by Goldberger to produce pellagra is deficient not only in  $\rm B_2$  but in other respects as well. Later observations have shown that his diet contains a sufficient amount of the Vitamin B complex to maintain normal growth in rats, although this might not suffice for man or the dog. The possibility of a multiple deficiency must be considered. There is much indirect evidence that other factors are also concerned in the development of the disease, but there is as yet no positive evidence as to what these may be. Ancylostomiasis, gastrointestinal disorders and other debilitating diseases are undoubtedly predisposing causes in certain cases (secondary pellagra).

Treatment.—A nutritious diet including liberal quantities of milk and beef is essential to prophylaxis and treatment. This should be supplemented by large amounts of yeast to supply more B<sub>2</sub>, and with liver or liver extract in severe cases. (Parenteral injections of highly concentrated liver extracts which are potent in pernicious anaemia are relatively inert in pellagra.)

## Scurvy

Scurvy was for centuries the scourge of seamen on long voyages during which their food consisted chiefly of salted meat and hard tack. It has exacted a heavy toll in all wars from the Crusades to the World War. although in the latter more from civilians than from the soldiers. The statement has sometimes been made that scurvy has been the most important factor in determining the outcome of wars. Documents as old as the records of Captain Cook's voyages show that the value of fresh food in scurvy was recognized, and in 1747 Lind introduced the use of fresh lemon and orange juice for the prevention and cure of the disease. Lime juice came into use later, but it is now known that it may contain little or no vitamin C, even when fresh.

Clinical picture.—The onset of the disease is insidious. There are vague complaints of languor, irritability, palpitation, dizziness and often fleeting pains in the legs. Dental caries is frequent and may be the only

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manifestation of the disease. Anaemia is often present (see p. 358). Many individuals whose diet is on the borderline of a vitamin C deficiency present only such indistinctive symptoms.

The earliest characteristic signs are sponginess of the gums with bleeding, fetor of the breath, and sometimes ulceration. The teeth are often loosened, and in extreme cases necrosis of the jaw bone may occur. There is a very characteristic tendency to capillary haemorrhage, and petechial spots about the hair follicles are very common. Haemorrhages into the mucous membranes and joints, epistaxis, and haematuria occur, and slight injuries are followed by large ecchymoses. Subperiosteal haematomata are characteristic; they are very painful and tend to break down and form ulcers. The ankles are usually swollen, and brawny indurations of the subcutaneous tissues develop. The urine may contain a large amount of albumin. The temperature is usually subnormal in uncomplicated cases. Anaesthesias and paraesthesias may occur. Mental changes, even delirium, may be present in extreme cases. In infants a rapid pulse and repiration due to an affect on the vagus has been noted.

Infantile scurvy (Barlow's disease).—In infants there is also a tendency to separation of the epiphyses from the shafts of the long bones, and any movement is exquisitely painful. A "scurvy rosary" similar to that seen in rickets may occur. Anaemia and asthenia are particularly marked. Hess has called attention to the frequency of latent cases in infants fed on sterilized milk. Irritability and lack of vigor may be the only manifestations, which clear up when orange or tomato juice is added to the diet.

Pathological lesions.—The essential pathological change is believed to be a failure of the capillary endothelial cells to form and maintain the intercellular cement substance. The formation of the connective tissue of the organs and teeth is also disturbed.

Scurvy in animals.—Guinea pigs are susceptible to scurvy and develop lesions which are very similar to those found in human cases. Definite lesions of the teeth appear by the second week. Young animals on a scurvy-producing diet require the addition of at least 10 units (0.5 mg. of cevitamic acid or its equivalent) daily to prevent the development of gross lesions. This diet in rats produces polyneuritis, but the bone lesions of scurvy. If peanut oil is added, scurvy develops. In fowls the same scorbutic diet produces polyneuritis.

Etiology.—In 1928 Szent Györgyi isolated from the suprarenal cortex of animals and from oranges and raw cabbage a substance, hexuronic acid, which was later found to be antiscorbutic and identical with vitamin C. The name of this substance has been changed recently from ascorbic acid to cevitamic acid. The properties of this vitamin and its content in

various foods are given in the tables. It is easily destroyed by cooking, from oxidation rather than heat. Orange, lemon and tomato juice and other foods can be concentrated to a syrup or dried with little loss of the vitamin if heated in a partial vacuum. Dried peas and beans contain no vitamin C but develop it when germinating. This fact has been utilized to obtain the vitamin when fresh food was not available. Cevitamic acid can be prepared synthetically.

Diagnosis.—In manifest scurvy in adults, which has become a rare disease in civilized communities, the diagnosis is easily established by the characteristic clinical picture and by the history of dietary deficiency. Latent cases, however, are common in both adults and infants, and their recognition is difficult. The symptoms are manifold but vague, and their dependence upon a vitamin C deficiency is established chiefly by the fact that they are relieved by the addition of the vitamin to the diet.

Hess utilized the capillary resistance test for diagnosis. A sphygmomanometer cuff is placed around the arm and a pressure of about 90 mm., sufficient to obstruct venous return, is maintained for three minutes. If scurvy is present, petechial spots will appear in the skin of the arm below the cuff within a few minutes (as in purpura). In latent cases the results are inconstant, however, and they do not furnish an accurate index of the degree of vitamin C deficiency. Dalldorf and Russell (1935) report that the test becomes negative promptly after the intravenous administration of 100 mg. cevitamic acid and remains so for at least 24 hours.

Quantitative estimations of cevitamic acid in the urine have been suggested as a diagnostic aid, particularly in latent cases. This substance is normally excreted in the urine in fairly constant quantities, about 30 mg. daily. In latent scurvy this may drop to 20 mg. or even to 15 mg.

The method depends upon the capacity of vitamin C (cevitamic acid) to reduce and decolorize solutions of dichlor-phenol-indo-phenol. The dye may be obtained in tablets, each of which corresponds to r mg. of cevitamic acid. (Hoffmann-La Roche.) (1) Dissolve one tablet in 100 cc. of distilled water, put 10 cc. of the solution in a small flask and add a few drops of glacial acetic acid. (2) From a burette add just enough of the solution (T cc.) to be tested to decolorize the dye solution. (3) The amount of cevitamic acid in mg. in 1 cc. of solution = 0.1/T.

The titration must be done rapidly. The dye solution must be freshly prepared. The urine must be titrated immediately after it is voided, or it must be acidified by adding one tenth its volume of glacial acetic acid, stored on ice in the dark, and titrated within a few hours. Urine contains other reducing substances which interfere to some extent with the reaction, and to get precise results they must be removed (as by precipitation with mercuric acetate, Emmerie and van Ekelen, Biochem. I., 1034).

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To avoid these difficulties Harris and others have suggested the following procedure. If a large test dose of cevitamic acid is given to a normal individual with adequate reserves of the vitamin, a large proportion of it is excreted promptly in the urine. If the individual is suffering from a deficiency of the vitamin, the latter will be retained. The patient is kept for three days on a diet restricted in fruits and fresh vegetables. Then in the morning from 200 to 600 mg. of cevitamic acid are given, and the excretion in the urine is measured as described. In normal cases the amount excreted is so large that the other reducing substances do not interfere. If only a small amount is excreted, the test should be repeated daily until a large excretion is obtained.

Parenteral administration of the vitamin can be carried out if absorption is disturbed by vomiting or diarrhoea. Cevitamic acid (in dose of 100 mg., neutralized with 50 mg. of sodium bicarbonate and dissolved in 10 cc. of physiological salt solution) may be given intravenously; or 15 to 35 cc. of orange juice which has been filtered, neutralized with soda, sterilized and diluted with about three parts of salt solution may be given every three days.

Relation to infection.—There is considerable indirect evidence that a deficiency of vitamin C lessens resistance to bacterial infection. It has been reported to detoxify diphtheria toxin in vivo and in vitro and it neutralizes poliomyelitis virus in a concentration approximately equal to that in which it normally occurs in brain substance (see Poliomyelitis). The requirements of the body for vitamin C are markedly increased during an infection. The amount excreted in the urine is markedly reduced. A manifest attack of scurvy may be precipitated in a latent case by an infection or even by vaccination.

Rand scurvy.—The scurvy endemic on the Rand in South Africa, as observed by Darling, is characterized by exaggeration of the knee jerks, hypertrophy and dilatation of the right heart and often vagal degeneration. These cases are closely related pathologically to beriberi, but they usually show spongy gums and a tendency to haemorrhage.

### RICKETS

Rickets is primarily a disease of infants although it may occur in children as old as five or six. It is more common in the dark-skinned races.

Clinical picture.—Digestive disturbances and fretfulness or apathy usually precede the characteristic symptoms. These are (1) a diffuse soreness of the body, (2) slight fever with nocturnal restlessness, and (3) profuse sweating, especially about the head and neck. The child does not begin to crawl or walk until later than normal and even then seems disinclined to move about. Weakness of the abdominal musculature causes the so-called "pot belly." Dentition may be delayed, and the teeth when erupted are often defective.

Bone changes.—The skull and the ribs are the first bony structures to show changes. During the fourth and fifth months the flat bones of the skull are thin and soft (craniotabes); later they show irregular thickenings with a characteristic proturberance of the frontal and parietal bones. The fontanelles are slow to close. The ribs become enlarged at the costo-

chondral junction (the rachitic rosary) and are depressed at the attachment of the diaphragm. Thickening of the epiphyses proximal to the wrists and ankles is very characteristic. The legs are apt to become deformed—bow-legs, knock-knees, saber-shins, etc.—after the child starts to walk.

Pathology.—The affected bones show characteristic chemical and anatomical changes. The inorganic matter, as measured by the total ash content, is greatly reduced even to 50% in severe cases. Even when the reduction in the total quantity of calcium and phosphorus is marked, however, the ratio between them remains the same as in normal bone. The histological picture is characteristic. The zone of provisional calcification at the junction of the epiphysis and diaphysis is absent, and the zone of proliferative cartilage is wide and irregular (the rachitic metaphysis). Blood vessels from the shaft penetrate into the latter zone and absorb the cartilage in scattered spots, and the orderly arrangement of the cartilage cells is completely destroyed. There is a compensatory overgrowth of uncalcified osteoid tissue in masses along the shaft and at the ends of the bones, giving rise to the visible enlargements. These changes are pathognomonic of rickets, and their demonstration by roentgenograms is an important point in the diagnosis of early cases. During convalescence the histological picture returns to normal and calcium salts are gradually deposited in an even, narrow line in the zone of provisional calcification. The McCollum-Shipley "line test" used in the assay of vitamin preparations is based upon the appearance of this zone after immersion of a longitudinal section of the bone in a solution of silver nitrate. The smallest amount of the unknown preparation which will produce a normal line in a rachitic rat within a given period (10 days) is taken as 1 unit.

Etiology.—Rickets is caused by a disturbance of the metabolism of calcium and phosphorus which is due to a deficiency of vitamin D. Ca, P. vitamin D and parathyroid hormone are all essential for the mineralization of normal bone, but the exact part played by each and their precise relationships to one another are not known. The action of parathyroid hormone in maintaining the level of Ca and P in the blood has been discussed in the chapters on Blood Chemistry and Endocrine Glands. It is generally believed that vitamin D acts by promoting the absorption of Ca and P or by regulating their excretion by the intestinal tract and kidney. The development of the disease depends also to some extent upon the ratio of Ca to P in the diet. If the P intake is low, absorption is blocked by the formation of calcium phosphate in the intestine (provided the supply of vitamin D is limited), and the disease can be cured in some cases simply by restoring the normal balance between them (two parts of Ca to one of P).

Some authorities believe that vitamin D acts by stimulating the parathyroids. Compensatory hyperplasia of the parathyroids has been described in rickets and also in osteomalacia, and Hamilton and Schwartz

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have reported an excess of parathyroid hormone in the blood of rachitic infants. The blood phosphatase is greatly increased (see p. 674).

In most cases of rickets the P content of the blood is reduced (below 3 mg.) and the calcium is approximately normal. Less frequently the Ca is reduced and the P normal, the so-called "low calcium rickets." If either element is deficient in the blood, normal deposition of calcium phosphate in the osteoid tissue does not occur. Howland has pointed out that when the product of the Ca content of the blood in milligrams by that of the P, the "solubility product constant," is below 30, rickets is invariably present, and it is usually present when the figure is 40 or less. The low calcium cases may be complicated by tetany. The amount of Ca and P in the faeces is high, and that excreted in the urine is low.

Administration of vitamin D restores the quantity of Ca and P in the blood to normal and increases the amount excreted in the urine. It relieves tetany, if this is present. Tetany is also relieved by the administration of parathyroid hormone, but this does not cure the rickets.

Treatment.—Rickets can be cured by the administration of vitamin D in any form provided the diet contains an adequate amount of calcium and phosphorus in suitable proportions. In severe cases irradiated ergosterol (viosterol) may be necessary in order to obtain a sufficient quantity of the vitamin in a palatable form. In mild cases the fish liver oils, irradiated animal or vegetable foods, or exposure to sunlight or ultraviolet light may effect a cure. In the latter case the vitamin is formed from the ergosterol normally present in the skin, probably in the fatty material of the sebaceous glands. Cod liver oil can be given parenterally, and it has been shown to be effective when rubbed into the skin. The prophylactic use of viosterol or cod liver oil is recommended, particularly during the winter months. Premature infants, twins and syphilitic infants are prone to develop rickets and should be given from 10 to 20 drops of viosterol daily for protection (Shelling and Hopper, 1936).

Osteomalacia.—This disease, which affects chiefly pregnant women, (3 to 5 times the normal amount of vitamin D is required in pregnancy) is believed to be similar to rickets etiologically. Both low phosphorus and low calcium cases have been described. Kay has noted an increase of the phosphatase in the blood which he ascribes to leakage from the affected bone. The disease responds to the usual anti-rachitic therapy.

Tetany.—Tetany in infants which is associated with a low Ca in the blood is also relieved by the administration of viosterol and Ca. In tetany due to parathyroid insufficiency viosterol should be given with caution, in small doses and with a diet high in Ca and low in P.

Idiopathic steatorrhoea (coeliac disease) is characterized by gastrointestinal disturbances which impair absorption, particularly of fats. There is also a reduction in the calcium in the blood and osteoporosis which is due to defective absorption of calcium salts and probably of viosterol from the intestine. The osteoporosis may be relieved by the administration of viosterol.

Hypervitaminosis.—Symptoms of hypervitaminosis have been induced in man by viosterol administration, but only by quantities at least 1000 times as great as the

usual therapeutic doses. The symptoms described include anorexia, gastrointestinal disturbances, pains in the joints and muscles, headache, dizziness, weakness, incoordination, loss of memory. Hypercalcaemia has been observed.

Nutritional Oedema (War Dropsy, Famine Oedema).—During the World War a condition characterized by weakness and oedema was observed in the parts of Europe where the shortage of food was most acute.

In certain countries, such as Haiti and Java, a form of nutritional oedema is prevalent among classes whose diet is for any reason inadequate. Among infants who live for long periods on a preponderatingly starch diet, striking degrees of dropsy are seen which appear similar to nutritional oedema in all respects.

Clinical picture.—The oedema is more marked than in ordinary cases of starvation. It is most prominent in the legs and feet. At times it extends to the thighs and trunk and, in about half of the cases, the face. Upon disappearance of the oedema, marked emaciation is apparent. Extreme muscular weakness and disturbances of the alimentary tract are common. Slight exertion is followed by dyspnoea and a slow pulse, although cardiac disturbance is not a feature of the disease. The urine is pale, with a low specific gravity, and free from albumin. There is an anaemia, with a tendency to leukopenia. Ocular manifestations due to a deficiency of Vitamin A were also noted in Central Europe where there was a shortage of fats.

Etiology.—Nutritional oedema is generally ascribed to an inadequate and unbalanced diet of low protein content. Deficiencies in mineral substances and in vitamins may also be important factors. The condition is aggravated by heavy physical work. Chemical studies of the blood show a decrease in the plasma proteins. A similar condition can be produced in animals by diets which are deficient in protein and certain mineral salts.

Treatment.—Treatment with high protein diets may bring about a prompt restoration of the plasma protein with recovery. In some cases, however, the response is slow, possibly from defective absorption or utilization of the protein.

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### A. APPARATUS

# THE MICROSCOPE

The most important piece of apparatus for the laboratory worker is a good microscope with a triple or quadruple nose-piece. A mechanical stage is almost a necessity in blood work and is useful in examining bacterial preparations. For some purposes, however, it is better to move the object with the fingers, and the stage should be readily removable. In addition one should have a magnifying glass or preferably a lens in a tripod or an achromatic triplet for the preliminary study of microscopic objects. A dissecting microscope is even better for this purpose and is almost essential in the examination and dissection of helminthological and entomological specimens. Of particular value is a stage forceps for handling insects.

The following precautions should be observed to prevent injury to the microscope:

- 1. If the fine adjustment works through the arm of the microscope, always grasp the instrument by the pillar which supports the stage. In those microscopes, however, which are not constructed in this way the arm has a handle portion made to serve in lifting the instrument.
- 2. Always keep the microscope in its case or covered with a bell jar or cloth when not in use in order to keep away the dust.
- 3. Care should be observed to keep all parts of the microscope from coming in contact with acids, alkalies, chloroform, xylol and alcohol.
- 4. Always use Japanese lens paper in wiping off the dust from dry objectives or the immersion oil from the 2-mm. one. Should one neglect to wipe off the oil from an oil-immersion objective, the dried oil can be removed by wiping with lens paper moistened with a drop of xylol or chloroform, but the cleaning should be done as rapidly as possible, with a final wiping off with dry lens paper, to avoid damage to the setting of the lenses. Throw lens paper away after using it once.

- 5. Lenses are very apt to deteriorate in the tropics. One should be careful to protect his instrument from the direct light of the tropical sun.
- 6. If any oil is used on the mechanical parts for lubrication, all excess should be wiped off to avoid the catching of dust or gritty particles.

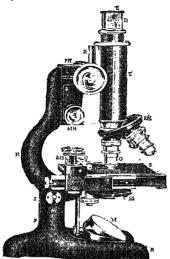


Fig. 100.—Parts of microscope. E. Eve-piece. D. Drawtube. R. Rack (coarse adjustment). PH, Pinion head. T, Body tube. MH, Micrometer head (fine adjust-

S, Stage. H, Handle, a part of the arm. SS, Substage. Mirror. B. Base.

Objectives.—To meet the demands of clinical microscopy there should be three objectives, preferably a 16-mm. (2/3-inch), a 4-mm. (1/6-inch) and a 2-mm. (1/12-inch) homogeneous oil-immersion. Some workers prefer a 1/5-inch objective to the 1/6-inch.

A dust-proof quadruple nose-piece with four objectives will be found a great convenience (in addition to the 3/3-inch and 1/12-inch objectives, a 14-inch for urine and blood counting, with a 1/2-inch for examining hanging-drop preparations and for quick examination of blood smears).

The Zeiss AA is a 17-mm, objective, and the Leitz No. 3, an 18-mm. or 34-inch one. The Zeiss D is about 4.2 mm. and the Leitz No. 6, 4.4 mm. or 16-inch.

Objectives are usually designated by their equivalent focal distance. The equivalent focal distance does not represent the working distance of an objective, by which is meant the distance from the upper surface of the cover glass to the lower surface of the objective. a 1/4-inch objective may have to be approached to the object so that the distance intervening may be only 1/6-inch or even less. This explains the frequent inability to focus an object when

is used with a rather thick cover glass-the objective possibly having a short working dis-I, Inclination joint. P. Pillar. M. tance, so that the thickness of the cover glass does not allow of any free working distance.

Numerical Aperture.—Objectives frequently have their numerical aperture marked on them. This is expressed by the letters N.A. From a practical standpoint this gives the relative proportion of the rays which proceeding from an object can enter the lens of the objective and form the image. Of course, the greater the number of rays, the greater the N.A., the better the definition, and consequently the better the objective. As a rule, a higher N.A. is gained at a sacrifice of depth of focus and also of working distance. Hence, in blood counting with the haemacytometer, the cover glass being comparatively thick, it may happen that with a 1/6-inch of high numerical aperture there may not be sufficient working distance to bring the blood cells into focus, which could be done with an objective of lower numerical aperture. Consequently, we must always consider the matter of working distance as well as that of numerical aperture.

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In addition to being marked with the equivalent focal distance in mm., the Spencer objectives are also marked with the N.A. in mm. and the magnification. The Bausch and Lomb objectives are marked only with the equivalent focal distance and the N.A. in mm. An apochromatic objective costs about three times as much as an achromatic one and, except in photographic work, has little if any advantage.

In using the oil-immersion objective a drop of cedar oil (or mineral oil) must be placed on the preparation before focusing. Cedar oil, having the same index of refraction (1.52) as glass, does not deflect rays coming from the object and so prevent their entering the objective, as would be the case if we used a dry objective with an intervening air space. In this case a portion of the rays would be turned aside because of the difference in the refractive index of air.

Oculars.—These are as a rule numbered according to the amount they increase the magnification given by the objective; thus a No. 2 increases the magnification, given by the objective alone, twice; a No. 8, eight times. The Spencer, Bausch and Lomb, and Zeiss oculars are marked in this manner. Oculars increasing the magnification given by the objective 5 and 10 times will be found to fulfill all ordinary requirements.

Some oculars are classified according to their equivalent focal distance and are referred to as ½-inch, 1-inch, and 2-inch oculars. A 1-inch or 25-mm. ocular increases the magnification produced by the objective about ten times while a 2-inch or 50-mm. one increases the magnification of the objective five times. A Leitz Huyghenian eyepiece No. o has an equivalent focal length of 62.5 mm. (2½ in.) and magnifies four times. The Nos. 1, 2, 3, 4 and 5 have an equivalent focal length of 50 mm. (2 in.), 41.65 mm. (1½ in.), 31.25 mm. (1½ in.), 25 mm. (1 in.) and 20.85 mm. (½ in.) respectively, which give eyepiece magnifications of 5, 6, 8, 10 and 12 times.

The oculars in common use are known as negative or Huyghenian oculars, by which is meant an ocular in which the lower lens (collective) assists in forming the real inverted image which is focused at the level of the diaphragm within the ocular. When using a disc micrometer, it is supported by this diaphragm, and the outlines of the image are cut by the rulings on the glass disc, and so we are enabled to measure the size of the object examined. The measurement of various bacteria, blood cells, and parasites is exceedingly simple; it assists greatly in the study of bacteria, and is indispensable in work in animal parasitology. When an ocular is termed positive, it refers to an ocular which acts as a simple microscope in magnifying the image, the image being formed entirely by the objective and located below the ocular.

By fixing one end of a hair on the rim of the diaphragm inside the ocular with a minute drop of balsam one has a satisfactory pointer to locate any particular cell in the microscopic field.

In order to get full advantage from apochromatic objectives, especially in photomicrography, it is necessary to employ with them so-called compensating oculars, which are designed to compensate for and correct the residual color defects in the extra-axial portion of the visual field. These oculars are useful with ordinary achromatic objectives also.

Tube Length.—The tube length is measured from the eye lens of the ocular to the end of the tube into which the objective is screwed. If a triple nose-piece is used the tube length must be shortened accordingly. As a rule objectives are corrected to use with a tube length of 160 mm.

Instrument makers generally specify the thickness of cover glass to be used with a certain tube length, but for all practical purposes it will be found satisfactory to use

No. 1 (very thin) cover glasses exclusively. The principal objection to these is that they are more fragile than the No. 2, but with a little practice in cleaning cover glasses this is negligible. Immersion lenses are less affected than dry lenses by the question of a certain thickness of cover glasses for a certain tube length.

Rule for determining the magnifying power of microscopic lenses: Measure the diameter of the lens of the objective in inches—the approximate equivalent focal distance is about twice the diameter. Dividing 10 by the equivalent focal distance gives the magnifying power of the lens. This should be multiplied by the number of times the ocular magnifies. Example: The diameter of the lens of the objective was found to measure ½ inch, the focal distance would then be about 1 inch. Dividing 10 by 1 we have 10 as the magnifying power of the lens of the objective. If we were using a No. 4 ocular, the magnifying power would be approximately forty.

Focusing.—When using the high-power dry or the oil-immersion objective it is very important to focus on the preparation in the following way. Lower the objective with the coarse adjustment until it is almost in contact with the cover glass or slide, controlling it with the eye on a level with the stage. Then, looking through the eyepiece, elevate the objective until the object comes into view, and focus sharply with the fine adjustment. Never use the fine adjustment to lower the objective. Contact cannot be felt and the cover glass is apt to break. This ruins the preparation and may injure the lens. Particular care is necessary in focusing hanging drop preparations since the cover glass is easily broken and there is the risk of infection if virulent organisms are present. It saves time to locate a suitable field for study with the low power (% inch) objective before using a higher power.

Illumination.—Proper illumination is very important in microscopical work; unless the light is utilized to the best advantage, the best results cannot be obtained. A north light, or, south of the equator, a southern light is desirable. Direct sunlight or an excessively bright light is to be avoided if possible or reduced by white shades or curtains. Special microscope lamps can be purchased with filters which give optical effects similar to those obtained with daylight. Many microscopists prefer to use these lights exclusively in order to obtain a uniform illumination at all times. In examining fresh blood preparations or hanging drops the concave mirror should be used and the light almost shut off by the iris diaphragm so as to give a contour picture. In examining a stained blood or bacterial preparation, the Abbé condenser should be properly focused so as best to illuminate the stained film. In many instruments set-screws are provided which check the elevation of the Abbé condenser when the proper focus is reached. Inasmuch as the light from the condenser should come to a focus exactly level with the object studied, in order to gain "critical illumination," it is evident that a fixed position for the condenser would not answer when slides of different thickness were used. It is best to use a slide about 1.5 mm. thick. Using daylight as described, or a special microscopists' lamp, the plane mirror should be employed when a color image is desired as in examining stained bacterial or blood films. If ordinary artificial light is used the concave mirror gives better results as such rays are not parallel. But by using a 6-inch globe filled with a very dilute solution of copper sulphate and a few drops of ammonia the rays are rendered practically parallel, and the light transmitted made to approximate daylight in quality, in which case the plane mirror should be used. Ordinarily in examining tissue sections, the Abbé condenser should be put out of focus either by racking down or by the use of the concave mirror and the narrowing of the aperture of the iris diaphragm. To examine the cells of a section with the oilimmersion objective, we use the condenser racked up fully and with the plane mirror instead of the concave one. It is only with the plane mirror that the rays are focused on the object by the condenser. Swing-out condensers are now made which are very convenient. The proper employment of illumination comes only with experience, and one should continue to manipulate his mirrors, diaphragm, and condenser until the best result is obtained.

Dark-field Illumination.—This is secured by the use of a special condenser, the center of which is covered by an opaque area so adjusted that no direct rays of light

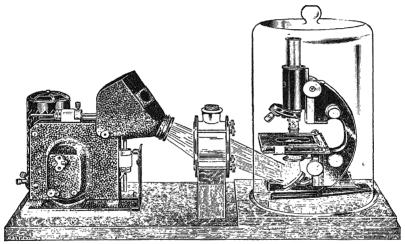


Fig. 191.—Dark-field assembly (Zeiss). This consists of an arc lamp, the carbons of which are automatically kept approximated by a clock-feed mechanism; a heat filter; and a microscope provided with a cardioid (dark-field) condenser. The parts are mounted on a special base-board to keep them in correct relative positions. If such an assembly is in daily use, it is practically necessary to have a microscope devoted exclusively to this purpose. (Courtesy of A. H. Thomas Co.)

from the lamp can enter the eye. The objects in the field are illuminated by light entering obliquely from the lateral portions of the condenser. Some of these rays are so reflected from the object that they reach the eye. The structures under examination thus appear as brilliant, silvery objects against a dark background. In this way particles less than  $0.2\mu$  (and beyond the range of resolution by direct illumination) may be made "visible" as points of light (ultramicroscopy, see p. 168). These are, however, diffraction images and not the actual objects.

With the lower-power objectives dark-field illumination can be secured by pasting a small, round piece of black paper, just large enough to block out direct light, in the center of a glass disc that fits in the ring under the lens of the (ordinary) substage condenser. For oil-immersion objectives the special condenser is required. For convenience this should be selected to fit the microscope with which it is to be used.

#### APPARATUS

Various manufacturers now provide excellent outfits which can be quickly substituted for the usual condenser.

A brilliant source of illumination is essential for satisfactory work. A small arc lamp is the most satisfactory, but a good gas light or even a strong flash light can be used in emergencies. The rays should be parallel when they enter the condenser. This can be secured by inserting a water-bottle condenser between the light and the microscope. In some recent types a light is incorporated in the apparatus below the condenser (Fig. 192). These are relatively cheap, compact and very convenient, in that they save the time and trouble involved in properly adjusting the relative distances between the light and water-bottle condenser and the microscope, necessary with the older types.

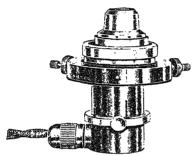


FIG. 192.—Dark-field condenser with illuminating bulb incorporated in the base. This can be substituted quickly for the usual sub-stage condenser in an ordinary microscope. (Courtesy of .1. H. Thomas Co.)

The light is less intense than that of an arc, however, and is less satisfactory for fine work.

If an ordinary oil-immersion objective is used, a special funnel-stop must be inserted in the objective in the lower end just above the lens to reduce the N.A. to 0.9 or less. This is not necessary with the special objectives designed for dark-field illumination work.

The condenser must be centered precisely. With the low-power objective observe the concentric rings usually placed on the upper surface of the condenser and adjust the set-screws until the rings are exactly parallel with the outline of the field. If the condenser is not marked with rings, one may (with the low power) put a dot of white ink in the center of the condenser, and then center this spot under the high power. Wipe off the dot before proceeding.

Put a drop of oil (or with some systems, glycerin) on the top of the condenser, put the slide to be examined on the stage, and raise the condenser until the oil touches the slide. There should appear in the center of the field either a bright spot or small circle. Rack the condenser up or down until a small spot of maximum brightness and sharpness is obtained. Re-center if necessary.

The thickness of the slide used must correspond closely to that called for by the condenser (usually 1.45 to 1.55 mm.), or it will be impossible to focus the condenser sharply. The slide and coverslip must be clean and free from scratches. The layer of fluid to be examined must be thin, and it must not contain too many objects or the whole background will be brightened and the objects indistinct. Air bubbles must be avoided. After centering and focusing the condenser with the low power, focus the oil-immersion lens on the preparation in the usual way. The method is especially useful in the detection of treponemata in material from hard chancres or mucous patches, of leptospira, of the spirochaetes of relapsing fever, etc.

### MICROMETRY

Micrometry, or the determination of the dimensions of an object microscopically, is often very useful in haematology, bacteriology, and particularly in animal parasitology.

The unit in micrometry is the *micron*. This is the  $\frac{1}{1000}$  part of a millimeter, and is usually written  $\mu$ . A *millimicron* is the  $\frac{1}{1000}$  part of a micron, or 0.000001 millimeters, and is written  $m\mu$  or  $\mu\mu$ .

Ocular Micrometer.—The most practical way of making these measurements is with an ocular micrometer which can be bought separately and used in place of the ordinary ocular. The disc micrometer is a glass disc with ruled lines which can be

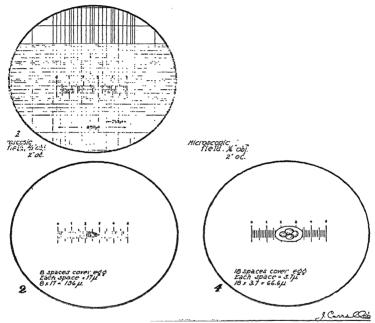


Fig. 193.—I. Fifty lines of ocular micrometer covering 17 small square spaces each) of haemacytometer. Each ocular micrometer space = 17 $\mu$ . 2. Schistosome egg fills 8 spaces of ocular micrometer. Egg is 136 $\mu$  long. 3. Fifty lines of ocular micrometer cover 3.75 small square spaces. Each space = 3.7 $\mu$ . 4. Hookworm egg fills 18 ocular micrometer spaces. Egg is 66.6 $\mu$  long.

placed on the diaphragm of the ordinary ocular with the ruled surface down. The image of the object is formed at the level of this diaphragm so that the lines of the image cut those of the lines on the disc. The most accurate instrument for measuring is the filar micrometer, but it is expensive. Measurements can also be made with a camera lucida, but it takes considerable time to make the necessary adjustments.

Before using a micrometer it must be standardized with each objective to determine the value of the spaces. All that is necessary subsequently in measuring is to count the number of lines or spaces which the image of the object fills and then, knowing the value of each space for that objective, to multiply the number of spaces by the value of a single space. The tube length must always be the same as that used in the standardization.

The ocular micrometer is usually ruled with 50 or 100 lines or spaces, separated by longer lines into groups of 5 and 10. It may be standardized with a regular stage micrometer if one is available. These have ruled lines separated from one another by  $\frac{1}{10}$  mm. (100 $\mu$ ). Some of these  $\frac{1}{10}$  mm. spaces are again ruled with 10 lines giving spaces which are only  $\frac{1}{10}$  mm. (10 $\mu$ ) apart. If one does not have such a scale, however, a haemacytometer makes a very satisfactory substitute. In any system of ruling of the haemacytometer, whether it be the Thoma-Zeiss, Türck or Neubauer type, there are small squares in the central ruling of crossed lines which are used for counting red cells. These are in groups of 16, and each one is  $\frac{1}{10}$  mm. or  $50\mu$  square.

Having focused the ruling of the haemacytometer we note the number of small squares covered by the 50 or 100 ruled lines of the ocular micrometer and multiply the number of squares so overlaid by 50, which gives the micron value of the entire ocular micrometer ruled space. To obtain the value of each space divide by 50 or 100 according to the number of lines of the ocular micrometer. To measure the egg of an intestinal parasite, for example, we simply focus on the egg and note the number of spaces covering it and multiply this number of spaces by the value in micra as of the space for the objective used.

Practical Points in the Use of the Microscope.—All preparations should be examined first with the low power objective to select suitable areas for further examination and to get all possible details. With tissue sections a preliminary study with a magnifying glass or even the unaided eye may give a surprising amount of information.

Position.—Although some workers prefer to use the microscope with the body tube inclined by the inclination joint, yet one gets just as good results by keeping the tube perpendicular and it is better to accustom one's self to such a position, because it is necessitated when we work with fluid mounts.

The eye.—It is advisable to cultivate the use of both eyes in doing microscopical work. When using one eye the other should be kept open with accommodation relaxed, since squinting of the unemployed eye causes fatigue. A strip of cardboard 4 or 5 inches long, with an opening to fit over the tube of the microscope, leaving the other end to block the vision of the unused eye, will prevent the strain. This apparatus can be purchased in vulcanite. Binocular microscopes are now made which are entirely satisfactory, although expensive. They have the advantage of affording stereoscopic vision, and give an appearance of depth to an object.

Care of the oil-immersion.—After using the oil-immersion objective the lens should be wiped clean. Dried oil on a lens often causes the lens to be considered defective. Accidental contact of the dry objectives with oil is not uncommon and should always be thought of when satisfactory optical effects are not obtainable. In depositing the drop of immersion oil on the slide bubbles are at times formed which make it almost impossible to use the  $\frac{1}{14}$ -inch objective. Under such circumstances I either prick the bubbles or wipe off the oil and deposit a drop anew.

Warm stages.—A warm stage for the study of living protozoa may be improvised by taking a piece of copper, about the size of the stage, with a strip projecting out anteriorly for 5 or 6 inches. The under surface of the plate is covered with flannel and a hole about 1 inch in diameter cut out of the center. The proper amount of heat is applied by a flame impinging on the tongue-like projection of the copper plate.

At present there are electrically heated, thermostatically-controlled warm stages, connected with the desk socket by a wire and plug, which are most convenient. In fact they are as satisfactory as the more expensive and less convenient warm chamber or microscope oven surrounding the microscope.

#### APPARATUS FOR STERILIZATION

Sterilizers.—For sterilizing glassware, media, etc., one of three methods is ordinarily employed, the hot-air sterilizer, the Arnold sterilizer, or the autoclave. Since the nutritive properties of certain albuminous fluids used for the cultivation of bacteria are impaired by heat, it is customary to sterilize such fluids, when necessary, by diluting and passing them through a Berkefeld or other bacterial filter. In practice, however, ascitic fluid or serum is preserved with aseptic precautions and hence needs no sterilization.

Hot-air Sterilizer.—The hot-air sterilizer is ordinarily used for the sterilization of Petri plates, test tubes, pipettes, etc. For this purpose a temperature of about 150°C is maintained for one hour. If the temperature is allowed to go too high, there is danger of charring the cotton plugs of test tubes and also, due to the development of an empyreumatic oil, of making the plugs unsightly and causing them to stick to the glass. We must be careful not to open the door until the temperature has fallen below 60°C.; otherwise there is danger of cracking the glassware. Where gas is not obtainable, the hot-air sterilizer is not a very satisfactory apparatus.

Arnold Sterilizer.—The Arnold sterilizer is to be found everywhere and can be used on blue-flame kerosene-oil stoves as readily as with gas burners. The most convenient form, but more expensive, is the Boston Board of Health pattern. The ordinary round pattern, with a telescoping outer portion, answers all purposes, however. In the Arnold, sterilization is effected by streaming steam at 100°C. It is usual to maintain this temperature for fifteen to twenty-five minutes each day for three successive days. The success of this procedure—fractional sterilization—is due to the fact that many spores which were not killed at the first steaming have developed into vegetative forms within twenty-four hours, and when the steam is then applied such forms are destroyed. Experience has shown that all the spores have developed by the time of the third steaming, so that with this final application of heat we secure sterilization. It is customary to use the Arnold for sterilizing gelatin, carbohydrate and milk media, even when the autoclave is at hand, the idea being that the greater heat of the autoclave may interfere with the quality of such media.

The Autoclave.—The most convenient autoclave is the horizontal type. The source of heat may be either electricity, gas, the Primus kerosene-oil lamp or steam from an adjacent boiler.

Sterilizing glassware.—Glassware will come out from such an autoclave with wrappers as dry and plugs of the test tubes as stopper-like as could be effected in a hot-air sterilizer. In sterilizing flasks, test tubes, Petri dishes, throat swabs, pipettes, etc., it has been our custom, after exposing to 20 pounds' pressure for twenty minutes, to produce a vacuum for two or three minutes, and then to leave the steam in the outer jacket for a few minutes to thoroughly dry the articles in the disinfecting chamber. The valve to the inner chamber is then opened to break the vacuum; the door is now opened, and the dried articles removed. Or they can be thoroughly dried in the

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sterilizer without the use of a vacuum by simply allowing the steam to remain in the outer jacket with the steam cut off from the inner chamber.

# PRESSURE AND TEMPERATURE TABLE

5 pounds pressure	107.7°C.,	227° F
10 pounds pressure	115.5°C.,	240°F
15 pounds pressure	121.6°C.,	250°F
20 pounds pressure	126.6°C.,	260°F
25 pounds pressure	130.5°C.,	267°F
30 pounds pressure	134.4°C.,	274°F

Tests conducted at the U. S. Naval Medical School showed that in order to obtain the above pressure-temperature relations the sterilizer chamber must be free and kept free of air during period in which steam enters chamber. This can be accomplished by keeping the chamber drain valve slightly but continuously open. Failure to expel air results in marked deviation from the normal pressure-temperature relation. For example: In one test in which the air was not expelled until the chamber pressure reached 20 pounds the temperature within the chamber at 10 pounds pressure was only 78°F, and at 20 pounds pressure only 149°F.

All such particles as Petri dishes, pipettes, swabs, etc., are wrapped in a cheap grade of filter paper, a fold being made and the ends turned in as is done in a druggist's package. Old newspapers answer well for this purpose.

Sterilization of Fermentation Tubes.—The regular type with a bulb and closed arm containing carbohydrate media for determining the gas formula of bacteria must be sterilized in the Arnold to avoid boiling over. If a gas analysis is not desired, however, the Durham tube is recommended. These can be sterilized in the autoclave using only a slight steam pressure if the carbohydrates are in the medium. (It is considered better to sterilize the sugars separately in 20% solutions and add measured amounts to the tubes just before using.)

Should a small bubble remain in the top of the small inverted inner tube after removal from the autoclave, one may make a mark with a grease pencil at the line of the bubble; or, if preferred, the basket of Durham tubes can be heated to boiling for ten minutes in a pan of water or in the Arnold and, after cooling, the bubble will be found to have disappeared.

The Durham Tube.—Into a test tube, about  $25 \times 175$  mm., we introduce the special sugar media, then drop down a small test tube ( $12 \times 75$  mm.) with its open end downward. Insert the plug of the large tube and sterilize. During sterilization the fluid enters the mouth of the smaller tube and fills it, and when the medium is subsequently inoculated, if gas forms, it appears in the upper part of the closed end of the smaller tube.

Drying Oven.—An electric drying oven is very useful, but is not indispensable for the ordinary clinical tests.

Inspissators.—For inspissating blood-serum slants a regular inspissator is desirable, this being nothing more than a double-walled vessel with the space between the walls filled with water.

As a substitute one may take the common rice cooker (double boiler) described in the chapter on media. Fill the outer part with water; and in the inner compartment pack the serum tubes properly slanted on a piece of wood or wedge-shaped layer of cotton. Place a weight on the cover of the inner compartment to sink it into the surrounding water. Boil for one or two hours. This same apparatus may be used for their sterilization, the process being repeated on two subsequent days, but it is better to sterilize in the autoclave or Arnold.

Centrifuge.—An electric centrifuge with a speed of 3000 or more r.p.m. is necessary to sediment bacteria and for many chemical and haematological procedures. Smaller instruments can be used for simple urine examinations. Water-power-driven centri-

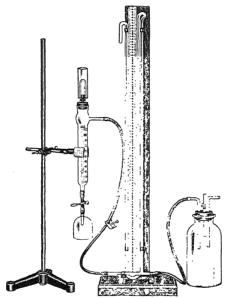


FIG. 194.—Filter apparatus (Mudd). This consists of a Berkefeld filter inserted by means of a rubber stopper in the upper end of a graduated suction-cylinder. This terminates in a delivery tube which is controlled by a pinch cock, and protected from contamination by a glass apron. A manometer controls the degree of suction applied. (Courtesy of A. H. Thomas Co.)

fuges are less satisfactory and hand ones least so. The gyroscopic centrifuges are very satisfactory.

Filter Pump.—A filter pump screwed into the water faucet or preferably to a water supply pipe, and connected with a vacuum gauge, is very useful for cleaning pipettes, removing the gastric contents, etc. It is indispensable for filtering material through the various types of Berkefeld and porcelain filters.

#### FILTRATION

There are several types of filters in common use for removal of bacteria from liquids.

Berkefeld Filters.—These are made of diatomaceous earth, pressed into the shape of a hollow candle and cemented into a metal base which is drawn out into a tube. This is inserted in a glass mantle which holds the fluid to be filtered, and is attached by means of a rubber stopper to an ordinary suction flask or to some special device as in Fig. 194. There are three grades of porosity: V (viel), coarse, (pores 8 to  $12\mu$ ) is for clearing solutions and does not retain all bacteria. N, normal, (pores 5 to  $7\mu$ ), retains ordinary bacteria. W (wenig), fine, (pores 3 to  $4\mu$ ), retains bacteria and some viruses.

Mandler Filters.—These filters, which are much used in the United States, are similar in construction and use to the preceding. They are made of diatomaceous earth,

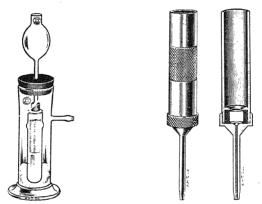


Fig. 195.

Fig. 196.

Fig. 195.—Filter apparatus (Martin). This consists of a Pyrex glass suction-cylinder with side arm, closed at the top by a rubber stopper; a large funnel-tube containing the liquid to be filtered, which is inserted in an opening in the large rubber stopper and is connected by means of a small rubber stopper with a Chamberland filter-bougie. The latter is inserted into a large sterile test tube, which collects the filtrate. (Courtesy of A. H. Thomas Co.)

Fig. 196.—Jenkins filter, external view and cross section, to show filter block in place. Capacity of metal cylinder about 50 cc. (Courtesy of A. H. Thomas Co.)

asbestos and plaster of Paris. There are three grades: "preliminary," "regular," and "fine," corresponding approximately to V, N, and W Berkefelds.

Chamberland Filters.—These are made of unglazed porcelain (kaolin with a little sand). They are pressed into candles open at one end, into which the stem of a funnel can be fitted by means of a rubber stopper (Fig. 195). The fluid filters through the candle from within outwards. There are nine grades: L I, (coarse, like Berkefeld V, not retaining bacteria), L I bis, L 2, L 3 (like Berkefeld N, pores 2.7 $\mu$ , retaining most bacteria), L 5, L 7, L 9, L 11, and L 13 (finest, retaining some viruses).

All of these filters are obtainable in various sizes.

Jenkins Filters.—These consist of a small, unglazed porcelain block 10 mm. in diameter, mounted in a cylindrical metal holder (Fig. 196). It has about the same porosity as a Berkefeld N candle. It is useful in filtering small amounts of fluid, since

only about 0.4 cc. is retained in the block. This can be cleaned by incinerating in a furnace or in the flame.

Seitz Filters.—These consist of a special asbestos pad which fits in a metal holder (Fig. 197). The grade EK ("germicidal") removes ordinary bacteria. The discs are discarded when exhausted.

Ultrafiltration through specially prepared collodion membranes is much used in the The technique is complicated. (See Elford, J. Path. and Bacteriol., study of viruses. 1031. XXXIV. 505.)

Testing Filters.—Gross defects (cracks, leaking joints) may be detected by immersing the candle in water with the closed end up and attempting to blow air through it.

If it seems intact, assemble the filter, sterilize in the autoclave and filter a liquid to which has been added sufficient of a 24 hour broth culture of prodigiosus (S. marcescens) or some similar organism to give about 100,000,000 organisms per cc. Culture liberal amounts of the filtrate. If no growth appears within 48 hours, the filter may be regarded as intact.

Cleaning Candles (Rivers' method).—If infectious material has been filtered, soak in some disinfectant such as cresol which does not coagulate protein. Scrub the surface with a brush, and force through the filter from within outward water (or salt solution if the fluid filtered contained globulin) until clean. Boil half an hour in 2% sodium carbonate, and then in several changes of distilled water. Force water through the candle until it is clean and all alkali has been removed. If clogged with organic material, Chamberland candles may be dried in a warm oven and gradually heated in a muffle furnace to a dull red heat, and then slowly cooled. Berkefeld filters often crack if so heated.

Filtration.—This is better carried out by suction than by pressure. Filtration should be rapid, but the negative pressure should not exceed 35 to 50 cm. Hg. The liquid to be filtered A segment has been should be cleared of detritus by preliminary centrifugalization cut away to show and filtration through paper, cotton or a coarse filter.

The filtrability of a particle depends only in part upon the relative size of the particle and the pores of the filter.

The composition and reaction of the liquid medium is equally important (see p. 167). If the medium is acid, the filters will usually retain small bacteria and some viruses which will pass through if it is slightly alkaline. Filtration of viruses is facilitated by suspending them in meat-infusion broth or in 10% serum rather than in salt solution, or by first drawing some sterile broth through the filter.

Incubators.—Automatically controlled electric incubators (38°C.) and paraffin ovens (60°C.) have largely supplanted other types wherever electricity is available. (Electric heating units with thermo-regulators which are adjustable to either temperature can be purchased separately for use in old gas-heated boxes. These are very satisfactory provided that the insulation is good and that the size of the heater is suited to the capacity of the box.) When only gas is obtainable for heating, a constant temperature can be obtained by the use of one of the various types of gas thermo-regulators. The Reichert type is the one in general use, although there are many features about the



Fig. 107.—Seitz (Uhlenhuth laboratory model). the Seitz pad in position. (Courtesy of A. H. Thomas Co.)

822 APPARATUS

Dunham and Roux regulators which are advantageous. If the pressure of the gas supply varies from time to time, it is essential to regulate this by the use of a gaspressure regulator (Murrill's is a cheap and satisfactory one).

It is probable that the Koch petroleum lamp incubator is the most satisfactory one where gas is not obtainable. It should be of all-metal construction, and not have a wood casing, on account of the danger from fire.

An incubator may be improvised by putting the bulb of an incandescent electric lamp in a vessel of water. The proper temperature may be obtained by increasing the amount of water or by covering the opening more or less completely with a towel. The test tubes to be incubated can be put into a fruit jar or tin can, which receptacle is

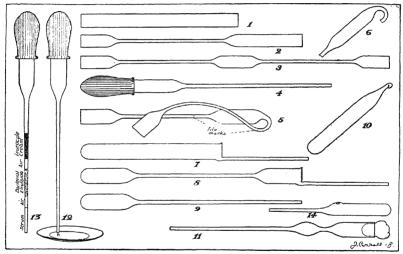


FIG. 198.—1, 2, 3, 5, 6, Wright's tube. 4, Rubber bulb pipette. 7, 8, 9, 10, Drawing out test tubes for stock vaccine. 11, Bulb bacteriological pipette. 12, 13, Pipettes for opsonic index work. 14, Lyon's blood tube.

placed in the vessel heated by the lamp. In emergencies we have used a chicken incubator for meningococcus plate work. Emery suggests the use of a Thermos bottle as an incubator. The vacuum bottle should be first warmed by pouring in warm water. Afterward the bottle should be three-fourths filled with water at roo°F. Schrup suspends his cultures and thermometer in the water by threads attached to pins in the cork of the vacuum bottle. The plug should be paraffined or covered with a rubber cap. There are now on the market large vacuum containers for keeping food warm. These may be used in transferring plate cultures from a distance to the laboratory incubator.

Room temperature incubators.—As regards the need of a low temperature incubator for gelatin work, this may be met by using a small refrigerator. The ice in the upper part maintains an even cold, and by connecting up an electric light in the lower part of the refrigerator we can easily maintain a temperature which varies only one or two

degrees during the twenty-four hours. The power of the bulb needed depends upon the size of the box, and must be determined by trial. The gelatin plates or tubes should be placed on the shelves usually provided with the refrigerator and not on the bottom.

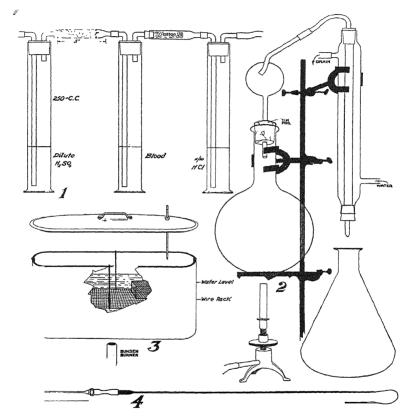


Fig. 199.—1. Apparatus to be connected with filter pump for blood urea determination. 2. Apparatus for distillation in making colloidal gold solution. 3. Substitute for expensive Wassermann water bath. 4. Capillary tube attached to needle for taking blood from vein in coagulation test.

We have used with entire satisfaction a low temperature incubator made by Hearson The low temperature is produced by water from cracked ice packed in a large centra! chamber. A small dynamo controlled by a thermostat circulates the water around the chamber containing the gelatin cultures. It requires some time for proper adjustment,

but afterward maintains a uniform temperature. Should the temperature of the room in which the incubator is installed fall below 22°C. there is provided an automatically controlled heating coil which then begins to operate.

Ice box incubator.—Certain laboratory procedures, for example the ice-box Wassermann technique, call for an incubation at a temperature of 6° to 8°C. It is difficult to obtain and maintain a temperature below 10°C with an ordinary ice box, and certain electrical refrigerators, while satisfactory in operation, are expensive. Armstrong of the U.S.P.H.S. Hygienic Laboratory has devised a simple, inexpensive ice chest for this purpose. It consists of an upper compartment for ice, and a lower, larger compart-

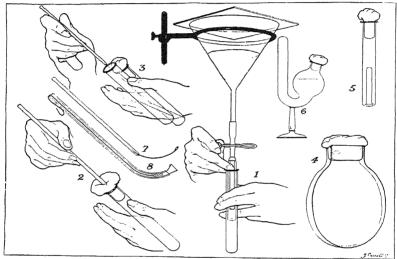


Fig. 200.—I, Filling tubes. 2, 3, Plugging tubes. 4, Culture flask plate. 5, Durham fermentation tube. 6, Smith fermentation tube. 7, Pharyngeal loop for meningococcus pharynx cultures. 8, West tube for same.

ment for refrigeration. The lower compartment is surrounded by a narrow air space continuous with the ice compartment. This permits water from the melting ice to drain down all sides, thus giving a maximum degree of refrigeration. The external construction and insulation are those of the ordinary ice box.

### CLEANING GLASSWARE

All glassware used in culturing bacteria should be sterilized in the autoclave before cleaning. This is a safe rule when dealing with dangerous pathogenic organisms (especially tetanus and anthrax). As soon as the tubes or dishes are taken out of the sterilizer they are emptied of their contents and placed in a 1% solution of washing soda and boiled. This cleans them thoroughly. As the washing soda raises the boiling-point of the solution slightly and also makes the spores more penetrable, it would be

sufficient to place all contaminated articles in a dishpan with the soda solution, and boil for at least one hour, as a substitute for sterilization in the autoclave. The tubes are next cleaned with a test-tube brush, thoroughly rinsed with tap water and placed in a 1% solution of hydrochloric acid for a few minutes; then rinsed thoroughly in water and allowed to drain over night. Some laboratory workers boil their test tubes and other glassware in water containing soap or soap powder and, after a thorough rinsing in hot tap water, drain. Hydrochloric acid should not be used after the soap as it will cause the formation of an unsightly coating difficult to remove. When thoroughly dry, tubes may be plugged and sterilized.

Cleaning Fluid.—A cleaning fluid commonly used in laboratories consists of I part each of potassium bichromate and commercial sulphuric acid with 10 parts of water. This is an excellent mixture for cleaning old slides, etc., especially when grease or balsam is to be removed. It is very corrosive, however. In cleaning glassware for such tests as the colloidal gold it is essential to use such a cleaning fluid. An efficient and less corrosive method for cleansing slides and cover glasses is to leave them over night in an acetic acid-alcohol mixture (two parts of glacial acetic acid to 100 parts of alcohol). After removing slides and cover glasses from this mixture, they are rinsed and dried; in addition, it is well to pass them through the flame of a Bunsen burner or alcohol lamp to remove every vestige of grease. The cover glasses should be exposed only momentarily to the flame to avoid warping. Ordinarily, rubbing between the thumb and forefinger with soap and water, then drying with an old piece of linen, and finally flaming will yield a perfect surface for making a bacterial preparation.

An excellent cleansing disinfectant is Liquor cresolis comp., U.S.P., which has a phenol coefficient of 1.87 to 3. A 5% solution is useful as a desk-jar disinfectant and in treating faeces, sputum, etc.

Capillary Pipettes.—With the possible exception of the platinum loop, there is no piece of apparatus so generally useful as the capillary pipette. It is made from a piece of ½-inch soft glass tubing, about 6 inches long. Held by the ends and constantly revolved, this is heated in a Bunsen flame, preferably fitted with a fish-tail tip, until it becomes soft in the center. It is then removed from the flame and, with steady traction, drawn out so that there intervenes a capillary portion 18 to 20 inches long. When cool, file and break off this capillary portion in the middle. We then have two capillary pipettes. By using a rubber bulb, such as comes on medicine droppers, we have a means of sucking up and forcing out fluids. The bulb should be pushed on about ½- to ¾-inch; this gives a firmer surface to control the pressure on the bulb.

A bacteriological pipette (Fig. 198, insert 11), is made from a piece of tubing 9 inches long. Heat successively in the flame points 3 inches from each end, in each instance drawing out the tube just sufficiently to make a constriction. Then, following the procedure described in the preceding paragraph, there are obtained 2 pipettes similar to the one illustrated. A piece of cotton is lightly pushed into the large end.

Wright's tube.—This tube, with a hooked end which permits hanging the crook on the centrifuge guard, is the best known apparatus for securing small quantities of blood for serum tests. By filing and breaking the thicker part of the tube, the serum is made directly accessible to a capillary rubber-bulb pipette, or to the tip of a haemacytometer pipette, thereby facilitating dilution of the serum.

Lyon's blood tube.—To make this tube, heat a 5- or 6-inch section of ½-inch tubing in the center and draw out as for making 2 bacteriological pipettes. Divide, and seal off the large end in the flame. Next seal off the capillary end. Then apply a very

small flame to a point on the large end just before it begins to taper to the capillary part. The heat causes the air sealed-off inside to force out a blow hole. To use, break off the sealed capillary end and allow it to suck up blood from a drop just as with the Wright tube. I consider this tube superior to that of Wright.

Although these pipettes may be sterilized during the flaming, and used immediately afterward, it is better to keep on hand a supply, suitably wrapped and autoclaved for use on occasion.

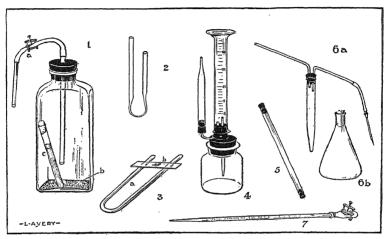


Fig. 201.—1, Apparatus combining various methods for culture of anaerobes; (a) Hoffmann clamp for connecting with vacuum pump; (b) pyrogallic acid at bottom of bottle for Buchner's O<sub>2</sub> absorption method; (c) deep glucose agar stab covered with sterile liquid petrolatum (see Anaerobes). 2, One-fourth inch capillary loop U-tube for making two nitric acid albumin tests (see Chemical examination of Urine). 3, Piece of tubing bent to hold slide for steaming smears in flame. 4, Schmidt's formentation apparatus, as modified by using graduated cylinder (see under Faeces). 5, One-fourth-inch glass tubing, 4½-inches long with corks at each end. For centrifuging faeces for ova. 6a, Apparatus connected with sterile centrifuge tube for taking blood from vein of man or a guinea pig or rabbit's heart. 6b, Erlenmeyer flask which can be used instead of centrifuge tube. See under sections Immunity and Blood. 7, A graduated pipette with Hoffmann clamp applied to rubber bulb for precise delivery of measured quantities of liquids.

Platinum Needles and Loops.—For use in making loops and needles, platinum wire of 26 gauge will be found most suitable. Handles made of glass rod are preferable to those of metal. One end is fused in the flame and, holding the 3- to 4-in. piece of platinum wire, with forceps, in the same flame, insert the glowing metal into the molten glass. By taking two lengths of platinum wire and twisting them together a more rigid needle is made for inoculating stab cultures. Loops and needles made from nichrome wire are almost as good as platinum ones and are very much cheaper. In making them, we saw a slit in aluminum rod handles and mash the nichrome wire in with a vise.

# EBONY DESK FINISH

#### USEFIII. HINTS

Ebony Finish.—A very popular method of preparing the wooden surfaces of laboratory desks, sinks, and tables is the application of the so-called "acid-proofing." This

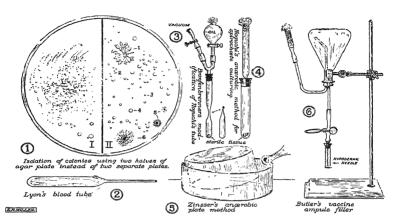


Fig. 202.-I, Method of using one plate instead of two for isolation of colonies (see page 12). The separated colonies on the No. II side of the plate are studied with unaided eye and achromatic triplet (1/2 in. or 3/3 in.) both by reflected and transmitted light. After we have determined the presence of two or more different kinds of colonies, a well isolated one of each type is selected and a blue pencil ring made around it on the glass surface of the back of the Petri dish together with a number. This number is carried along on culture tubes or microscopical slide preparations until the organism is identified. 2, Lyon tube for blood. More convenient than the Wright tube. For description see page 825. 3. Bronfenbrenner's anaerobic tube. This is made by drawing out a single test tube instead of using two separate tubes as with the Noguchi method. Before drawing out the test tube the piece of sterile tissue is introduced and after drawing out in the flame the ascitic broth or sheep serum water, as well as the material for culturing, is introduced through the drawn-out neck with a bulb capillary pipette. The lower part of the tube is placed in water at 37°C. and the vacuum connection made; after exhaustion of air the sterile paraffin oil is run in. The drawn-out portion is then sealed off in a small flame and looks like a sealed vaccine ampule. 4, Noguchi apparatus. 5, Zinsser method for anaerobic plates (page 831). 6, Butler's apparatus for filling vaccine ampules. The sterile vaccine is put in the sterile flask, and the stopper, with air intake and needle filler separately sterilized, then introduced into neck of flask which is then inverted.

gives an ebony-like finish which is not affected by strong acids. In using it, the surface of the wood must be new, or freshly planed, so as to be free of varnish, oil or paint.

# Solution I

Ferrous sulphate	20 Gm
Copper sulphate	20 Gm
Potassium permanganate	40 Gm
Water, sufficient to make	

Apply two coats of this solution with an interval of at least twelve hours between applications. When thoroughly dry apply two coats of solution No. 2.

### Solution 2

Aniline oil.	60	cc.
Hydrochloric acid	()0	cc.
Water, sufficient to make	500	cc.

When the treated surface is thoroughly dry apply one coat of raw linseed oil with a cloth. After this is dry wash with very hot soapsuds. The excess of the black comes off during the first few days following application.

An aspirator bottle on a shelf elevated 2 feet, with rubber tubing and glass tip leading to a small aquarium jar or other desk receptacle, makes a good substitute for a small sink and faucet. A Hoffmann screw-clamp on the rubber tube controls the flow of water. The glass tube passes through a wooden clothes pin clamped on the edge of the jar.

Ordinary glass salt cellars will be found very useful as substitutes for laboratory watch glasses, or, after sterilization, to hold fluids for inoculating, etc.

A glass-topped fruit jar or a specimen jar containing a disinfecting solution for contaminated slides, etc., should be on every working desk. A good solution is that of Harrington (corrosive sublimate, o.8; commercial HCl, 60.0 cc.; alcohol, 640.0 cc.; water, to 1000.0 cc.).

There are many excellent types of water bath incubators on the market for use in Wassermann work. As a substitute in field work one may take an ordinary oval wash boiler and suspend in it, by wire supports bent over its edge, a heavy wire gauze diaphragm. Test-tube racks containing the Wassermann tubes are supported on this diaphragm. The boiler is filled with water up to the desired height above the supporting diaphragm. Once the temperature of the water is brought up to 37.5°C. the temperature can be maintained by a small flame under the wash boiler. For details see Fig. 199.

Plugging Test Tubes.—To plug a test tube, pick out a little pledget of plain absorbent cotton about 2 inches in diameter from a roll. Place it over the center of the tube and with a glass rod push the cotton down the tube about an inch. In culturing slow-growing organisms, as tubercle bacilli, or certain pathogenic protozoa, it is necessary to have plugs so prepared as to prevent drying out of the medium in the tube. The simplest way of accomplishing this is to melt some paraflin in a pan, then removing the cotton plug with the fingers to dip the end entering the tube into the melted paraflin and then replace. Plasticine, sealing wax or paraflin may be used to seal over the tops of such test tubes. We have found that impregnation of the cotton plugs with vaseline answers as well in keeping cultures from drying out as paraflin.

Sterile Swabs.—The sterile swab can be used for many purposes in the laboratory. It is most easily made by taking a piece of copper wire about 8 inches long, flattening one end with a stroke of a hammer, then twisting a small pledget of plain absorbent cotton around the flattened end. Wooden applicators may be used. After wrapping, the swabs are sterilized in bunches or singly in stoppered test tubes. We not only use them for getting throat cultures, but in addition for culturing faeces, pus, or other such material. The material obtained with such a swab is then distributed in a tube of sterile broth or water. With the same swab the surface of an agar plate is stroked in a series of lines.

Gas Substitutes.—Where gas is not at hand, the Barthel alcohol lamp gives a flame similar to that of the Bunsen lamp and is equally satisfactory for heating glass tubing. By making a collar with a lateral opening to fit the burner of a Primus lamp a powerful side-flame is obtained which is almost as suitable for glass blowing as the Bunsen blast usually employed. The ordinary plumbers' blast lamp also can be used.

One cannot appreciate the importance of a gas supply for a laboratory until he has experienced the lack of it. At present electricity is generally available but it cannot replace gas.



Fig. 203.—"Selfblo" automatic alcohol torch.

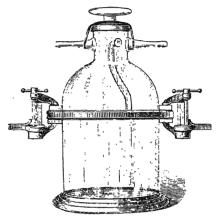


Fig. 204.-Novy jar.

In the Philippines we had a Tirrill gasolene gas generator for 50 burners. The apparatus costs only about \$500 and gives absolute satisfaction. It includes a tank for the gasolene, placed about 40 feet from the building, and a mixer and air pump which are placed in the cellar of the laboratory.

The Matthews gas machine, which weighs complete 1600 pounds, is quoted at 326.00 for a 50 burner plant.

## Anaerobic Methods

Novy Jar.—This is one of the most convenient and satisfactory devices for securing strict anaerobic conditions. Any one of several methods described may be used for abstraction of oxygen. A good substitute may be improvised from an ordinary museum jar, the top of which is perforated to admit a rubber stopper carrying one or two glass tubes through which air can be withdrawn and hydrogen introduced. (A glass stopper is better.)

The method of McIntosh and Fildes or one of its modifications is now regarded as giving the most satisfactory anaerobic conditions obtainable. This method depends upon the oxidation of hydrogen by the oxygen in the jar due to the catalytic action of platinized, or preferably palladinized, asbestos wool. The culture tubes or plates are

put in the jar. The asbestos wool, protected by a small cage of copper gauze, is heated red hot and suspended in the jar from the lid. The jar is quickly closed tightly, and sealed with plasticine or some substitute. A stream of hydrogen from a cylinder provided with a good reducing valve is then introduced so that it passes over the asbestos wool very slowly to avoid an explosion. This is continued until the oxygen is exhausted, and the inlet tubes are then closed. As an indication that an adequate degree of anaerobiosis has been secured these investigators suggested using a tube of sterile 2% dextrose alkaline broth tinged with methylene blue. This is decolorized (reduced) when the oxygen is abstracted, and regains the blue color when oxygen is admitted.

Simple replacement of air by hydrogen from a cylinder or from a Kipp generator serves fairly well if palladinized asbestos is not available. It is more effective alter-

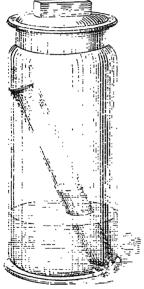


Fig. 205.—Arrangement of tubes for cultivation of anacrobes by Buchner's method. (MacNeal.)

nately to exhaust the air from the jar with an ordinary suction pump, and replace with hydrogen several times.

Buchner's Method.—In this method I gram each of pyrogallic acid and caustic potash or soda for every roo cc. of space in the vessel containing the culture is used to absorb the oxygen. It is convenient to drop in the pyrogallic acid; then put in place the inoculated tubes or plates; then quickly pour in the amount of caustic soda, in a 10% aqueous solution and immediately close the containing vessel. A large test tube in which a smaller one containing the inoculated medium is placed, and which may be closed by a rubber stopper, is very convenient. A good rubber-band fruit jar is satisfactory. A desiccator may be used for plates.

A Combination Method.—By combining various methods, as illustrated in Fig. 201, very satisfactory anaerobic conditions can be obtained. First, a deep tube of freshly-sterilized glucose agar is made and inoculated. This tube is put in a salt-mouth bottle in which an adequate amount of pyrogallic acid has been deposited. The rubber stopper provided with the glass and rubber tubing is then firmly pushed in and the apparatus is connected with a vacuum pump.

After 5 to 10 minutes, when almost all the air has been exhausted, the Hoffmann clamp is screwed up tight and the bottle is disconnected from the vacuum pump. The end of the glass tubing is then inserted into a vessel containing 10% NaOH. The Hoffmann

clamp is unscrewed, and when the necessary amount of caustic soda solution has run in, as indicated under the Buchner method, the clamp is screwed tight and the jar is incubated. This method may be used with a flat-sided culture flask in which the medium, such as glucose or blood agar, has been allowed to solidify on one side. The surface of the culture medium is then inoculated and the flask placed with the culture medium uppermost. After exhausting the air, run in first a 50% solution of pyrogallic acid and next 10% NaOH solution.

Zinsser's Method.—Zinsser has originated a satisfactory method for plate cultures of anaerobes, which is shown in Fig. 202.

Secure two small crystallizing dishes, about 3 and 4 inches in diameter by 1 inch in depth and sterilize. Pour the inoculated agar into the smaller of the dishes, or smear the surface of poured glucose agar with the material to be plated out. In the bottom of the larger dish place the dry pyrogallic acid, then invert the smaller dish with the agar surface over it. Quickly pour a 5% solution of caustic soda into the space separating the sides of the inverted smaller dish and the upright larger dish, to a depth of ½ inch, and while it is dissolving the pyrogallic acid, very speedily superimpose paraffin oil on the soda solution to make an air-tight seal.

J. H. Wright's Method.—Make a deep stab culture in glucose agar or gelatin, preferably boiling the media before inoculating. Then flame the cotton plug and press it down into the tube so that the top lies about three-fourths of an inch below the mouth of the test tube. Next fill in about one-fourth of an inch with pyrogallic acid; then add 2 or 3 cc. of a 10% solution of caustic soda, and quickly insert a rubber stopper. This method is one of the most convenient and practical, and is to be strongly recommended.

A simple method for obtaining anaerobic conditions with plates, either in a desiccator with the pyrogallic acid and caustic soda, or—less satisfactorily—in the open air, is to sterilize the parts of the Petri dish inverted; that is, the smaller part is put bottom downward in the inverted cover (as one would set one tumbler in another). Then, in using, unwrap the Petri dish, lift up the inner part, and pour the inoculated medium into the upturned cover. Then immediately press down the inner dish, spreading out a thin film of the medium between the two surfaces.

Vignal's Method.—In this, a section of glass tubing (14 in.) is drawn out at either end, so that there remain two narrowings of the lumen with an intervening section of full diameter. One end, which is to be used as a mouth-piece, is plugged with cotton; the other is broken off at the point of constriction. The liquid agar or gelatin is then inoculated and the medium drawn up into the tube by suction with mouth or better with a rubber bulb. In a very small flame the capillary narrowings are sealed off, and we have inside the tube satisfactory anaerobic conditions. To get at the colonies, file and break the tube at the desired point.

Tarozzi's Method.—In this method, pieces of fresh sterile organs are added to broth. Pieces of kidney, liver, or spleen are best suited. After adding the tissue the media may be heated to 80°C. for a few minutes without interfering with the anaerobic-producing properties of the fresh tissues. This is also a feature of Noguchi's method of culturing Treponema pallidum.

To get effective anaerobiosis it is necessary to put the pieces of tissue in the bottom of deep tubes of plain or glucose agar or broth, and to pour over the top sterile paraffin oil, or preferably melted vaselin or paraffin. Liquid oil retards but does not prevent diffusion of oxygen into the medium. Such tubes can be put in a Novy Jar and the oxygen largely exhausted by replacement with hydrogen or by the pyrogallic acid method.

Theobald Smith recommended using (for tetanus) fermentation tubes containing ordinary broth into which a piece of the liver or spleen of a rabbit or guinea pig has been introduced at the junction of the closed arm and the open bulb. By this method spores develop rapidly in from 24 to 36 hours. Sporulation is most rapid at 37°C. As there is always a possibility of a postmortem invasion of the viscera by ordinary saprophytes,

care should be taken not to handle the animal roughly in chloroforming and in pinching off pieces of the organ at autopsy. The animal must be healthy and the tubes to which the piece of tissue is added must be proven sterile by incubation.

The Method of Liborius.—In this it is necessary to have a test tube containing about 4 inches of a 1% glucose agar. Glucose acts as a reducing agent and furnishes energy. It is convenient to add about 0.1% of sulphin digotate of soda, the loss of the blue color at the site of the colony enabling us to distinguish it. The tube of agar should be boiled just before using to expel remaining oxygen from the tube. Then rapidly bring down the temperature to about 42°C., by placing the tube in cold water, and inoculate with the material to be examined. A second or third tube may be inoculated from the first, just as in ordinary diluting methods for plate cultures. Having inoculated the tubes, solidify them as quickly as possible using tap water or ice-water. The anaerobic growth develops in the depths of the medium. Some pour a little sterile vaseline or parafin or additional agar on the top of the medium in the tube as a seal from the air. Others have recommended the inoculation of some aerobe, as B. prodigiosus, on the surface. This latter method is not advisable. A deep stab culture is often sufficient.

The same technique can be applied to gelatin cultures for anaerobes, pouring in at the completion of the inoculation an inch or so of melted glucose agar to act as a stopper for the gelatin below.

Partial Oxygen Tension.—By incubating such tubes without sealing with oil or paraffin, gradations of partial oxygen tension are obtained from aerobic conditions at the surface to practically complete anaerobiosis at the bottom. Some strains of streptococci, e.g., grow only in a restricted zone below the surface.

Note.—To obtain material for examination and isolation in pure culture from the deep agar stab-tube, it is best to loosen the medium at the sides of the tube with a heated platinum spud or a flattened copper wire. Then shake the mass out into a sterile Petri dish. It is dangerous to break the tubes with a hammer as some do. With those anaerobes which produce gas in glucose agar, the split in the column of medium enables one to introduce a fine sterile capillary pipette to the site of a colony and by releasing pressure on the rubber bulb to draw up into the tip of the tube material for investigation.

Rockwell's Method for Growing Anaerobes.—When only a few anaerobes are transferred to artificial media they fail to grow if the oxygen and respiratory CO<sub>2</sub> are absorbed by pyrogallic acid and caustic alkali, but will grow upon removal of oxygen provided some CO<sub>2</sub> is present. In order to insure the presence of CO<sub>2</sub> in the culture tube the following solution should be used in place of caustic alkali:

Sodium bicarbonate	50.00 Gms.
Acid sodium phosphate	0.75 Gms.
Water	
Keep tightly corked.	

This method is particularly useful in growing parasitic streptococci. Cultures are made on dextrose ascites agar; the cotton plug is shortened by clipping with scissors and the plug pushed into the test tube to the upper level of the medium; a tight wad of absorbent cotton is placed on top of this; and then is added 0.5 Gm. pyrogallic acid and I cc. of the charged alkali solution and the test tube is corked tightly with rubber stopper.

Often when the material contains several bacteria the growth obtained in this way will, in 24 hours, show a pure culture of streptococci.

Carbon dioxide in 10% concentration is required for the isolation of Brucella abortus. The cultures may be placed in an air-tight jar of known capacity, the air in the jar partly exhausted, and a measured amount of CO<sub>2</sub> may be introduced from a tank, later equalizing the pressure.

If one or two actively growing cultures of B. subtilis are placed in the jar, approximately this amount of CO<sub>2</sub> is obtained.

The following simple method has been used successfully in the U. S. Naval Medical School. For a container use a two-quart Mason fruit jar with a one-hole rubber stopper into which is fitted a piece of glass tubing extending nearly to the bottom of the jar. Close the top with a short piece of rubber tubing with a clamp close to the glass. In the bottom of the dry jar containing the culture tubes, put 0.75 Gm. of anhydrous sodium bicarbonate. Stopper tightly. Partially evacuate jar with Chapman pump. Add through the glass tubing with a small funnel 5 cc. of 40% aqueous monosodium acid phosphate (NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O). After the reaction is complete (in about 15 minutes) place in the incubator and after an hour equalize the pressure in the jar.

#### B. CULTURE MEDIA

Most pathogenic bacteria require for their metabolism complex food-stuffs similar in composition and reaction to the fluids of the animal body. In general, a suitable medium for their cultivation contains moisture, salt, protein (amino acids) and a definite concentration of ionizable hydrogen. Infusions or watery extracts of meat furnish the basis of most culture media. Certain bacteria require enriching substances, such as haemoglobin, blood serum, carbohydrates or vitamins for artificial cultivation outside the body.

The consistency of media is modified by the addition of suitable amounts of gelatin, agar or albumin; thus giving a semisolid or solid medium, depending upon the amount of the various ingredients.

Dehydrated Culture Media.—Culture media in dehydrated form with directions for preparation and sterilization can be purchased (Difco Laboratories, Inc., Detroit, Michigan). Most of them require only the addition of distilled water, and their reaction is so adjusted that the pH after sterilization is suitable for growth. The pH is recorded on each container, and alterations can be easily made if desired. We have found these media very satisfactory and recommend them, especially for small laboratories with limited facilities. Even for larger laboratories the uniformity in the composition of these media is a substantial advantage.

Laboratories using only small amounts of culture media will find it convenient and economical to sterilize it in bottles with air-tight screw caps. These keep the media sterile and moist for a long time.

Media such as broth, agar, and potato should be sterilized in the autoclave at 10 to 15 pounds pressure for 15 minutes. Milk should be sterilized with the Arnold, subjecting it to three steamings for twenty minutes on three successive days. Gelatin may be sterilized in either way, but preferably in the autoclave at 7 pounds' pressure for fifteen minutes. As soon as taken out of the sterilizer it should be cooled as quickly as possible in cold water. This procedure tends to prevent the lowering of the melting-point of the finished gelatin and also preserves its spissitude.

In making media a rice cooker, or the apparatus described below, is almost essential. As it is necessary to make the contents of the inner compartment boil, the boiling point of the water in the outer compartment must be raised. This is done by using a 25% solution of common salt or of calcium chloride in the outer compartment instead of plain water. Should CaCl<sub>2</sub> be carried over to media in the inner compartment (as by thermometer) coagulation of albumin and clearing of media will be prevented. A 15% solution of salt raises the boiling point 2.5°C.; a 20%, 3.5°C., and a 25%, 4.5°C. Calcium chloride in solutions of similar strength raises the boiling point to about the same degree.

Makers of apparatus for the bacteriological laboratory now furnish a vessel for making media which has two bottoms with an intervening air space. This hot-air layer prevents the scorching of the media which is so apt to occur when a plain sauce-pan is used. Its advantages over the rice cooker are that time is saved in bringing the media to a boil, and it is easier to maintain a brisk boiling temperature.

### Reaction of Media

The method of adjusting the reaction of media by means of titrable acidity has been superseded by that based upon determination of the hydrogen-ion concentration.

It has long been recognized that there is an optimum reaction of media for each organism, and certain of them require great exactness in this respect. It has been particularly in cultivating this latter class that the defects of the older method have become evident. The inadequacy of the older method is due to several factors. The titration end-point with phenolphthalein in bacteriological media is not easy to recognize, and it varies in practice in different laboratories. A certain tint once secured means a definite pH, but it is the usual practice to secure the final, desired reaction by the addition of acid after neutralization or by the use of less alkali than is necessary to neutralize. Results can be consistent only as long as the medium is prepared from exactly the same materials and by precisely the same technique. The consequence of variability is that this final adjustment results practically in a very indeterminate pH, and the final media are by no means universally comparable. Titration while hot greatly increases the variability. We will, however, give the older method for such as still employ it.

The expression "per cent acid" (or "alkaline") indicates the reaction of media and its degree expressed as the number of cc. of N/I acid (or alkali) which, added to 100 cc. of the media at the neutral point, would produce a reaction in kind and degree equal to that existing. Acidity is also represented by a plus sign preceding, and alkalinity by a minus sign. Thus a 0.5% alkaline medium is one the alkalinity of which corresponds to that produced by the addition of 0.5 cc. of N/I NaOH to 100 cc. of neutral medium and is represented by -0.5. Note that here, as elsewhere, perfect accuracy requires that the amount of broth taken should be the volume desired minus the amount of acid, or alkali, to be added.

Hydrogen-ion Concentration.—See page 682 for a brief discussion of this subject. It is now generally recognized that this is the most accurate method for adjustment of the reaction of media.

The Barnett-Chapman method (see p. 879) for determination of pH, although not the most accurate, is serviceable for the adjustment of the reaction of media.

Sterilization practically always changes the pH of media. Heating in poor glassware accounts for a small part of the effect, but most is due to factors in the media, and the

total change is practically always an increased acidity. Sugar media show the greatest alteration. The greatest effect will occur after a previously alkaline pH (7.8–9.0), less with acid media (5.0–6.2), and that with neutral media (6.6–7.4) is practically negligible. The usual change is less than 0.2, and the maximum to be anticipated is about 0.4. Agar media are most conveniently adjusted in the broth stage, the later addition of agar causing no appreciable change. A rough adjustment of pH can be obtained by using the old titration method in connection with different indicators. The medium is titrated in the cold to a certain end point, but no acid or alkali is then to be added after neutralization in the vain expectation of securing a certain pH. One simply adjusts the whole batch as indicated by the titration of the sample. The first faint greenish-blue with brom-thymol blue would mean a pH of about 6.2; the first faint pink with phenol red, cresol red, or phenolphthalein would mean about 7.0, 7.4, and 8.4 respectively. Other end points can be devised from a study of the list of indicators on page 876. The reaction of the newer media described on the succeeding pages is given according to the pH method.

Titration (Hot).—Take 10 cc. of the medium with a pipette and let it run out into a porcelain dish. Add 40 cc. of distilled or rain water and about six drops of a 0.5% phenolphthalein solution. (Phenolphthalein, o.5 Gm.; dilute alcohol, 100 cc.) Bring the contents of the porcelain dish to a boil and continue boiling for one or two minutes in order to expel all CO2. Now from a burette filled with decinormal sodium hydrate solution run in this solution until we have the development of a faint but distinct pink which is not dissipated on further boiling. Having obtained the light pink coloration we read off the number of cc. or fractions of a cc. of N/10 sodium hydrate solution added to produce the color. This number gives the acidity of the medium in percentage of N/r acid solution. If we took 100 cc. of the medium and put it in a beaker and then ran in N/r NaOH solution from a burette, it will be readily understood that if we had to add 3.5 cc. of N/I NaOH to obtain the pink color, it would show that the acidity of the 100 cc. of medium being tested corresponded to 3.5 cc. of N/1 acid solution, and that its acidity was equal to 3.5% of N/I acid solution, or that its reaction was +3.5. As N/1 NaOH solution is too corrosive for general use in a burette, and as 10 cc. of medium is more convenient to work with than 100 cc., we use a solution one-tenth the strength of the N/I NaOH and we take only one-tenth of the 100 cc. of medium. In this way the same result is obtained as if we had used 100 cc. of medium and N/1 NaOH solution. The Λ. P. H. Association recommends 5 cc. of the medium and the use of N/20 NaOH; but as the N/10 NaOH is always at hand for titrating gastric juice, it is more commonly used.

Titration (Cold).—The recommendations of the A. P. H. Association call for making the titration with the medium boiling, but should it be found difficult to carry on the titration while boiling, the end reaction may be fairly accurately determined in the cold. Deliver into a beaker from a pipette 10 cc. of the medium and make up to 50 cc. with distilled water and add 5 drops of 0.5% phenolphthalein solution. Then run in N/10 NaOH from a burette and continue to add the N/10 NaOH solution from the burette, drop by drop, until further addition fails to show any intensifying of the purplish-violet color at the spot where it came in contact with the diluted medium in the beaker. This marks the end reaction and corresponds to the faint pink of the end reaction obtained at the boiling point. A delicate pink obtained in the cold indicates about +0.7. Having determined the percentage acidity of the 10 cc. sample tested, we easily calculate the number of cc. of N/1 NaOH solution required to be added to the 1000 cc.

#### CULTURE MEDIA

of the medium to obtain a reaction corresponding to the neutral point of phenolphthalein. It is more exact to take the average of two titrations. As 100 cc. of medium would require 3.5 cc., 1000 cc. would require 10 times as much, or 35 cc. N/1 NaOH solution. Having measured out and added 35 cc. of the N/1 NaOH solution to the meat infusion, containing salt and peptone, we have a solution which is exactly neutral to phenolphthalein, or 0. It is usually considered that a reaction of about 1% acid is the optimum reaction for bacterial growth. Hence we should now add 1% of N/1 HCl solution to the medium. This would be accomplished by adding 10 cc. of N/1 HCl solution to the 1000 cc. of neutralized medium, and we would have a medium with a reaction of +1. If we desired a reaction of 1% alkalinity we would add an additional cc. of N/1 NaOH solution to every 100 cc. of the medium at 0, or 10 cc. for the 1000 cc. of medium. The reaction would then be -1.

As a matter of convenience, the usual method followed is to determine the reaction of the medium and, it always being more or less acid, to add only enough N/r NaOH to reduce the acidity to the percentage desired, instead of neutralizing all the acidity present and then, in a second operation, restoring the acidity to the point desired. Thus, finding the acidity of the medium to be 3.5% and desiring to give it an acidity of 1%, we would add only 2.5 cc. of N/r NaOH to every 100 cc. of medium or 25 cc. for the 1000 cc. of medium. The reaction would then be +r. The neutral point of litmus is not a sharp one, but it corresponds rather closely with a reaction of +r.5 to phenolphthalein.

Thymol blue as indicator for the titration of media.-McIntosh and Smart have proposed the use of thymolphthalein (thymol blue) as an indicator with titration carried on at room temperature. The first development of a dirty greenish blue color seems to correspond with the pink of the phenolphthalein at boiling, and is a more easily obtained end reaction, the checking of results being more constant. Another end point, obtained by adding about r cc. more of N/r NaOH per litre, is represented by the appearance of a blue color that does not intensify upon the further addition of alkali. Measure 10 cc. of the medium into a porcelain evaporating dish; add 25 cc. of pure distilled water. Have a control dish alongside prepared in exactly the same manner. Add 5 drops of a 0.5% alcoholic solution of thymolphthalein to the first Then run in N/10 NaOH from a burette, stirring continuously, and looking for a darkening of color as compared with that of the control dish. Just before this point a precipitate of phosphates occurs. The point may be more precisely described as the disappearance of the yellow tint and the development of a bluish. Note the quantity of N/10 NaOH required to produce this change. In practice it is best to make four estimations, and the first in which a distinct blue has been produced is rejected. With the average data a calculation is made as to the amount of N/I NaOH necessary to produce the same reaction in the rest of the medium. This amount of NaOH is then added and the flask shaken.

The alkaline medium is now brought to boiling to deposit the phosphates and then filtered free from them. The reaction is now adjusted as desired. The addition of 10 cc. N/I HCl per liter of broth will give a reaction of approximately +1.

Clearing and Filtration of Media.—Albuminous material in culture media is coagulated at 65°C. and the fine particles in liquid media are removed by filtration through paper. Precipitates of phosphates in media may be removed by filtration after the media are cold, but these in no way interfere with bacterial growth. When eggs are used

to clarify, the whole egg or dried egg albumen is used. One egg is used to each liter, shaken or well beaten with an equal amount of water and stirred into the cooled medium. With dried egg albumen use 10 Gms. dissolved in 20 cc. of water and add to one liter of medium. The temperature of the medium is gradually raised to boiling over the free flame or in the Arnold for about one hour.

Media which solidify on cooling are usually filtered through absorbent cotton in a glass funnel kept warm by a water jacket or in the Arnold sterilizer. A good grade long fiber absorbent cotton is essential so that the lengths of cotton may be split horizontally and the split portions placed crosswise and placed on a square of gauze or preferably coarse wire netting. This prevents the cotton from jamming the neck of the funnel and gives a broad filtering surface. The cotton is moistened with the medium and the first filtrates returned until it comes through clear. The coagulated albumins settle on the cotton and act as a part of the filter.

Filtration through paper pulp is carried out in the Buchner funnel using a suction pump. Filter paper is shredded and placed in a wide-mouth bottle, using 6 large sheets of filter paper to 2½ liters of hot water. Shake vigorously at intervals for 48 hours until a fine uniform suspension is obtained. When ready, fit the bottom of the Buchner funnel with surgeons' lint, fleecy side uppermost. Dilute 400 to 500 cc. of pulp with 2 or 3 liters of hot water and pour carefully an even layer on the lint about one-fourth inch think and cover with a sheet of filter paper. Place a suction flask under the funnel and apply the suction pump to draw the water into the flask until the paper is firm, but moist. Now add the hot agar slowly to prevent breaking the filter and discard the first 100 cc. which contains the water from the pulp. No egg is used when media is filtered by this method as it clogs the filter.

By sedimentation or by use of the Sharpless super-centrifuge all contact with paper or cotton is avoided in clearing and the "hormones" or "vitamins" are retained in the medium. (See Huntoon's medium.)

Litmus Solution.—Litmus is used less than formerly, since the newer indicators described below are more satisfactory. Brom-cresol purple has about the same pH range as litmus and can be substituted for it in any of the media. A simple solution may be made by digesting the powdered litmus cubes repeatedly with hot water, mixing the extracts, and, after allowing them to stand all night, decanting the solution from the inert sediment into a clean bottle. A solution so made, however, contains not only the essential blue dye but also a red dye together with soluble calcium and other salts, and, while answering the demands of routine work, is not adequate for bacteriological purposes. In this work, there should be used a solution of the blue dye in a pure form called "azolitmin."

It can be conveniently prepared as follows: Weigh out 2 ounces of powdered litmus; digest repeatedly with fresh quantities of hot water until all the coloring matter is dissolved out; allow to settle, and decant the fluid from the insoluble powder. Add together the extracts, which should measure about a liter. Evaporate down the solution to a moderate bulk, then add a slight excess of acetic acid, so as to convert all carbonates present into acetates. Continue the evaporation, the later stages over a water bath, until the solution becomes pasty. Add 200 cc. of alcohol, and mix thoroughly. The alcohol precipitates the blue coloring matter, while a red coloring matter, together with the alkaline acetate present, remains in solution. Transfer to a filter. Wash out the dish with alcohol and add this to the filter. Wash the precipitate on the

filter with alcohol. Dissolve the pure coloring matter remaining on the filter in warm distilled water and dilute to 500 cc. Azolitmin solution prepared in this way is more sensitive than ordinary litmus solution.

Azolitmin in powder can be purchased from dealers in chemicals. It is used as a reagent in 5% aqueous solution neutralized with N/1 NaOH, 1 cc. of this solution being added to 100 cc. of liquid sugar media or of Russell's double-sugar agar.

Andrade's Indicator.—This indicator has come into general use as a substitute for litmus indicator for demonstrating acid production in carbohydrate media. To prepare it, take of acid fuchsin 0.5 Gm. and distilled water 100 cc. To this magentared solution add N/1 NaOH until the color changes to pink, then to brownish red and then to yellow. Shake the reagent after each addition of the alkali. Usually it takes about 17 cc. of normal sodium hydrate solution to decolorize 100 cc. of the fuchsin. Add 1 cc. of the decolorized reagent to 100 cc. of any sugar broth. It will be colorless at room or incubator temperatures, but pink at 100°C. If an organism produces acid the indicator will turn magenta-red.

Newer Indicators.—Bromcresol purple, phenol red and cresol red are the most useful indicators as these may be added directly to the medium in the minute quantities necessary without being reduced by bacterial growth as are litmus and methyl red. The choice of the indicator depends upon the initial reaction of the finished medium, and as bacterial growth proceeds, the changes in the pH values of the medium may be studied and recorded by comparing with a known standard from day to day. The amount of the indicator used in the medium is unimportant so long as a distinct color is obtained, as these indicators do not affect the bacterial growth. We have found that I cc. of a 1.6% alcoholic solution to a liter of the medium is usually satisfactory. When making comparisons with the standard pH tubes proportionate amounts of the indicator are used.

#### Nutrient Broth

This may be made from either fresh beef or veal, or from meat extract. Media from fresh meat are usually lighter in color and possibly clearer. Distilled water is generally used but rain or tap water can be substituted for most purposes.

Meat Infusion Broth.—Take 500 Gm. of minced round steak or veal, and add 1000 cc. distilled water. Mix and let stand in ice-box for 24 hours. Heat in streaming steam for 1 hour. Squeeze through cheese cloth until 1000 cc. is obtained. Filter through paper and make up loss with water. Add 1 per cent peptone and 0.5% sodium chloride, C.P. Heat in streaming steam for 30 minutes, determine reaction, and correct to pH 7.6. Filter through paper and place in test tubes, about 10 cc. to each tube, and sterilize for 2 hours in streaming steam, or in autoclave 15 minutes at 15 pounds pressure.

Meat Extract Broth.—Mix 3 grams of Liebig's extract, 10 grams of peptone and 5 grams of sodium chloride. Dissolve the whites of one or two eggs in 1000 cc. of distilled water and add, little by little, stirring constantly. Pour into the inner compartment of a rice cooker, and boil for 15 or 20 minutes. (The outer compartment should contain the salt or calcium chloride solution.) Filter and sterilize. The egg can be omitted.

The reaction of this medium is fairly constant and generally within the range suitable for most bacteria. Consequently it is not necessary to adjust the reaction unless unusual precision is required.

Giblet Broth.—Liver, spleen, etc., may be used for making broth. The technique is the same as for beef or other meat, substituting 500 Gms. of the solid organs for the meat. Very often these broths exhibit a slight cloudiness, but this cannot be avoided.

Hartley's Digest Broth.—Various "digest" media have been devised in which the requisite uncoagulable nitrogenous substances (peptones, amino acids, etc.) are derived from the meat by a process of digestion with pancreatic (or gastric) ferments. The following medium is very satisfactory.

Ox heart or lean beef free from fat and minced	150 Gm.
Distilled water	250 CC.
Heat to 80°C. and add	
Sodium carbonate (anhydrous) o.8% solution	250 CC.
Cool to 45°C. and add	Ü
Cole and Onslow's pancreatic extract	5 cc.
Chloroform	
Incubate at 37°C. for 6 hours, stirring frequently. Add con-	
centrated hydrochloric acid	
Boil for ½ hour, cool and filter through paper.	
Adjust the reaction to pH 7.6-7.9. Boil ½ hour. Cool and	L
filter through paper.	
Autoclave at 15 lbs. for 15 minutes.	
(For digest agar add 2% of agar to the above broth.)	

### add 2 /0 or agai to the above broth.

Fresh pig pancreas free from fat and minced	500 Gm.
Distilled water	1500 cc.
Alcohol 95%	500 cc.
Place in a large stoppered bottle and allow to stand three	
days at room temperature. Shake repeatedly.	
Strain through gauze and filter through paper. Add con-	
centrated hydrochloric acid in the proportion of o.1%.	
This causes a precipitate which soon settles out. The	

COLE AND ONSLOW'S PANCREATIC EXTRACT

This extract keeps indefinitely in a stoppered bottle.

extract can then be filtered.

Nutrient Agar Agar.—Take 1000 cc. of broth having a reaction of pH 7.6 and add a sufficient amount of N/1 NaOH to correct reaction to pH 8.0. Then add 15 Gm. of threaded agar, heat in streaming steam for 2 hours, or autoclave 30 minutes under 15 pounds pressure. Let stand in sterilizer overnight. Slip the cylinder of agar from the container, cut off the precipitate and discard it. Melt the clear portion and fill into test tubes, 5 cc. for slants and about 10 cc. for plates and stabs. Sterilize in streaming steam for 2 hours or autoclave 10 minutes at 15 pounds. The final reaction of this medium will be about pH 7.6.

Instead of threaded agar powdered agar may be used, but the following steps are required: Sprinkle the powdered agar on the surface of the cool broth; let stand until the agar has sunk to the bottom; stir briskly, heat in streaming steam for 2 hours and cool the medium to 60°C. Constantly stirring, add the white of one egg beaten up with an equal amount of water, mix thoroughly, heat in streaming steam for one hour, then filter through a moist paper in a hot sterilizer.

Ordinary agar on the market is a variable product and there is no way of telling its solidifying quality unless each lot is tested for its *pectin* content, which should be not less than 99%.

Test for Pectin.—Eight to ten Gm. of threaded agar is washed rapidly in running cold water and dried overnight at 37° to 56°C. Then weigh out 5 Gm. and dissolve in 1000 cc. of distilled water containing 2% sodium chloride, place in Arnold or autoclave for one hour. When cool add 2000 cc. of 95% alcohol to precipitate the pectin. Let stand overnight, filter through weighed filter paper and dry the residue in incubator at 37°C. Reweigh the residue and paper, calculate the amount of residue and calculate the percentage of pectin.

Nutrient Gelatin.—Take 1000 cc. of broth and 125 Gm. of "Best French Gold Label" or Nelson's photographic gelatin. Heat the broth and add the gelatin, stirring constantly. When the gelatin is in solution titrate and correct reaction to pH 7.6. Cool to 60°C. and add the white of one egg beaten up with an equal amount of water. Heat in streaming steam for 30 minutes, filter through paper and place in test tubes, about 10 cc. to each tube. Sterilize by the intermittent method, i.e., for 30 minutes in streaming steam on each of three successive days. Cool rapidly and store in a cool place.

Agar Gelatin (North).—This is meat infusion agar with 20 Gm. of gelatin (Gold Label) substituted for 5 Gm. of the agar.

Potato Medium.—Cut with a cork borer or apple corer a cylindrical piece of potato about 6 cm. long. Cut this in halves diagonally lengthwise and place in cold running water for one hour. Place the potato pieces in large test tubes, each tube containing a wad of cotton saturated with water on the bottom. Sterilize in streaming steam for 2 hours.

Potato Agar and Glycerin Potato Agar.—Grind up 1000 Gm. white potatoes, add 1000 cc. water and cook until thick soupy liquid results. Strain through gauze and make up to 1000 cc. with distilled water. Add agar agar to make 3 per cent. Autoclave at 15 pounds pressure for 30 minutes. Filter through cotton and gauze. Tube. Autoclave for 15 minutes at 15 pounds pressure. Slant. This media may be used for all purposes for which glycerin potato slants are used by addition of 5% glycerin.

## Sugar Media

Various sugars, alcohols, glucosides, etc., added to a sugar-free broth, are used to test the fermentative abilities of different bacteria.

These media are made by dissolving 1 or 2% of a sugar in sugar-free broth or that made from meat extract. Tube in Durham's or the ordinary fermentation tubes and sterilize in the autoclave at only about 5 pounds pressure for fifteen minutes, or in the Arnold. It is now considered essential that the sugar solutions, in 20% strength, be sterilized separately and then added to the sterile broth. 0.5 cc. of the 20% solution added to 10 cc. of medium would give a 1% solution.

Too high a degree of heat may turn the sugar broth brownish. The nature of the sugar itself may further be affected by too high a temperature, since many of the carbohydrates, when in broth, are apt to be split up on subjection to any marked sterilization. Maltose is particularly unstable. Inulin usually contains resistant spores so that its sterilization may need the autoclave. It is a polysaccharid resembling starch but does not give the iodine reaction. It is obtained from the roots of chicory or dandelion. The sugars used must be chemically pure. For further notes on carbo-

hydrates see chapters on the study and identification of bacteria and on water analysis. When it is desired to determine percentage of acid or lowered pH value produced by an organism in carbohydrate media do not add indicator until making titration or comparing with standard pH tubes.

Nitrate Broth.—Dissolve with the aid of heat 10 Gm. of peptone (Difco) and 1 Gm. of potassium nitrate (nitrite-free) in 1000 cc. of distilled water. Filter and sterilize in the autoclave at 15 lbs. for 15 minutes.

Sugar-free Broth.—Take 500 Gm. of minced round steak, free from fat, and 1000 cc. of distilled water. Mix and let stand in a cool place for 24 hours. Strain through cheese cloth until 1000 cc. of fluid are obtained. Inoculate the meat juice with 10 cc. of a 24-hour broth culture of *E. coli* and place in incubator at 37°C. for 24 hours. Heat in streaming steam for 1 hour. Filter through paper and make up loss with water to original volume. Correct the reaction to pH 7.6. Add 1 per cent peptone. Heat in streaming steam for 30 minutes and again titrate and correct the reaction to pH 7.6. Filter through paper and sterilize for 2 hours in streaming steam.

For ordinary purposes the very small amount of sugar in broth made from Liebig's meat extract may be neglected in determining gas production; the various sugars may be added directly to the meat-extract broth. Dunham's peptone solution may be used as a substitute for sugar-free broth, particular sugars being added as desired. We usually employ the serum water medium of Hiss, but it must be remembered that serum contains glucose (blood sugar), so that for critical work this medium should not be used.

Enlow's Sugar-free Medium for Fermentation Studies .- Take

Distilled water	1800.0 cc.
Peptone	10.0 Gm.
Na <sub>2</sub> HPO <sub>4</sub>	14 5 Gm.
KH <sub>2</sub> PO <sub>4</sub>	
Agar	2 0 Gm.

These substances are heated together in the Arnold sterilizer for 40 minutes, or autoclaved for 10 minutes at 15 pounds, filtered through paper while hot, and diluted with hot distilled water to 2000 cc. The salts added as indicated make adjustment of the reaction unnecessary for most organisms. The pH will vary from 7.2 to 7.4. The sugars, alcohols, glucosides, etc., are added in 0.5% amounts, followed by the indicator, after which the medium is tubed and sterilized by the fractional method (30 minutes in Arnold sterilizer on 3 successive days). The bromthymol blue is added in amount sufficient to give a distinct green-blue or deep-blue color (depending upon the pH desired). If the indicator is prepared as given below, 3 drops in each 500 cc. is a sufficient amount to give a very positive color change due to increased hydrogen-ion concentration.

The indicator may be prepared as follows:

Bromthymol blue (	dibromthymol	sulphonphthalein)	0	ı Gm.
N/20 NaOH			3.	2 CC.

Grind in agate mortar. When solution is complete add 3 cc. of distilled water. The salts and peptone tend to stabilize the medium, but the buffer strength is not sufficient to prevent appreciable change in the hydrogen-ion concentration from fermentation of the sugars. If a higher or lower pH value is desired, the salts can be varied according to Sørenson's table.

As a control, it is necessary to prepare one lot of tubes without fermentable substances, that is, using merely the water, peptone, potassium and sodium salts, agar and indicator. In case the peptone is broken down in such a way as to simulate carbohydrate fermentation this will be indicated in these tubes, which we have termed "non-fermentable controls."

Cooked Meat Medium for Anaerobes.—Take one part of ground up meat and two parts of tap water. Mix and heat in Arnold sterilizer from r to 1½ hours. Filter through filter paper and allow to stand for r hour. Distribute meat into large sized test tubes. Adjust reaction of broth to pH 8.5. Distribute broth into test tubes containing the meat, so that the broth and meat are about equal in volume. Sterilize 1½ hours at 15 pounds pressure. Add r to 2 cc. sterile vaseline. Heat r½ hours in Arnold sterilizer. Final reaction is about pH 6.8 to 7.0.

Calcium Carbonate Broth.—When we wish to cultivate such organisms as streptococci and pneumococci in massive cultures we may add small fragments of marble (calcium carbonate) so that any inimical excess of acid may be neutralized. Addition of a small piece of calf's brain and 0.5% dextrose as used by Rosenow is quite satisfactory.

Glycerin Broth.—Add 6% of glycerin to ordinary broth. It is used chiefly in the cultivation of tubercle bacilli.

Peptone Solution (Dunham's).—Dissolve 1% of peptone and 0.5% of sodium chloride in distilled water. Filter, tube, and sterilize. If the solution is too acid correct the reaction to pH 7.6. This medium can also be used for fermentation tests by adding various sugars.

Cholera Red and Indol Tests.—The former test is made by adding from 6 to 8 drops of concentrated  $\rm H_2SO_4$  to a 24 to 48 hour-old peptone-solution culture of the organism to be tested. (Each lot of peptone should be tested with a known cholera vibrio, since certain lots will not give a reaction.) If the organism is a true cholera vibrio, both indol and a nitroso body are produced and we obtain a violet-pink coloration, "cholera red." If no pink color is produced, add about 1 cc. of a 1–10,000 solution of sodium nitrite. If the organism belongs to the indol producing group the solution will now turn pink.

Another satisfactory method is to add to a three-day growth in plain broth, r cc. of concentrated HCl (or any strong mineral acid). Mix thoroughly and overlay the acid broth with r or 2 cc. of a 0.1% solution of sodium nitrite. At the junction of the two solutions a brownish-red ring develops if indol is present.

The Vanillin test also gives satisfactory results. Five drops of 5% vanillin solution in 95% alcohol and 1 cc. of strong sulphuric acid are added to about 5 cc. of the culture to be tested. The presence of indol is indicated by the formation of an orange color; the presence of tryptophan by a reddish-violet.

The following test for indol is the one most often used at present. Add r or 2 cc. of Ehrlich's reagent to an equal quantity of the culture. If indol is present a rose color appears which may be separated out by shaking with amyl alcohol. The reagent consists of paradimethylaminobenzaldehyde, 4 Gm., ethyl alcohol (95%) 380 cc., Concentrated HCl, 80 cc.

Medium for Methyl Red Test.-Take:

Distilled water	800 cc.
Difco peptone	5 Gm.
Dextrose	5 Gm.
Dipotassium hydrogen phosphate, K2HPO4	5 Gm

Heat with occasional stirring over steam for 20 minutes, filter through paper, cool to 20°C., and dilute to 1000 cc. with distilled water. Fill in test tubes, about 10 cc. to each tube, and sterilize by the intermittent method for 20 minutes on three successive days.

For preparation of the methyl red indicator solution and use of the test see page 204. Hiss' Serum Water Medium.—Take one part of clear beef serum and add to it about three times its bulk of distilled water. Heat the mixture in the Arnold for fifteen minutes to destroy any diastatic ferment which might be present. Add I cc. of I.6% bromcresol purple (or other indicator) and I% of the sugar to be used. Sterilize in the Arnold by the fractional method. Occasionally a serum will need to be diluted 4 or 5 times to prevent coagulation on heating. Some workers substitute I% peptone water or nutrient broth for the distilled water.

Glucose Agar.—Add the agar to 1 or 2% glucose broth and proceed as for ordinary agar. If preferred, the glucose agar can be made by rubbing up meat extract 3 grams, peptone 10 grams, salt 5 grams, glucose 10 grams and 15 grams of agar in 1000 cc. of water containing the white of one to two eggs, then boiling in the rice cooker and filtering.

Glycerin Agar. - Add agar to 6% glycerin broth, instead of to nutrient broth, or the glycerin may be added to nutrient agar which has been melted. Glycerin agar makes an excellent base for blood and serum media for use in culturing delicate pathogens.

Glycerin Agar Egg Medium.—Take the white and the yolk of one egg and mix thoroughly with an equal amount of glycerin agar in a vessel kept between 45° and 55°C. Tube the medium, inspissate in a rice cooker as for serum tubes, and sterilize as for blood-serum tubes.

This makes an excellent medium for growing tubercle bacilli. As egg medium has a tendency to be dry, it is well to add 1 cc. of glycerin broth to each slant before autoclaving.

Litmus Milk.—Milk for media should be as fresh as possible. As soon as received, it should be put in a 1000-cc. Erlenmeyer flask, sterilized for fifteen minutes in the Arnold, and set over night in the refrigerator. The next morning the milk beneath the cream should be siphoned off, care being taken that the short arm of the siphon does not reach the bottom of the flask so as to avoid including sediment. Add sufficient litmus solution to this milk to give a decided lilac tinge (or, preferably, brom-cresol purple); tube and sterilize in the Arnold on three successive days.

Litmus milk which apparently is as satisfactory as the above as regards nutritive quality and cultural characteristics can be made from certain canned milks which have not been condensed or sweetened and which do not contain chemical preservatives. The "Natura" brand of milk is the one with which I have experimented.

Löffler's Serum.—Beef blood is collected in sterile containers at the abattoir and placed in the cold storage room over night. The next morning the serum is pipetted off. If it is to be kept for a long time, it is advisable to add about 2% of chloroform and stopper tightly. This will preserve the serum and eventually sterilize it. Fildes recommends the following method. Five cc. of ether is added to each roo cc. in a bottle with a ground glass stopper. After fixing the stopper securely in place, the mixture is heated for one hour in a water bath at 45°C., and then placed in the incubator at 37°C. for several days, by which time it should be sterile. Before using the serum the ether is driven off by heating at 45°C. This is better than the old method using chloroform.

To make Löffler's serum, take one part of glucose broth and three parts of the serum Mix, tube and congulate in the inspissator, slanting the tubes before heating. This

requires an hour or two. Sterilize on the following day in the autoclave at 7 lb. If this is done on the same day, many of the slants will be disrupted by bubbles and ruined.

The autoclave as an inspissator.—When large numbers of blood-serum tubes are to be inspissated it is more convenient to use an autoclave. A screen frame is made to fit the inner chamber of the sterilizer and set at the desired angle for proper slant. The tubes are put in and steam is turned into the outer chamber only, never permitting the heat to rise above 3 pounds or 105°C. During the process of inspissation, which usually requires from 45 to 60 minutes, the door of the sterilizer should be opened occasionally in order to keep the temperature below the point at which bubbles are generated in the media.

The rice cooker as an inspissator.—The preparation of blood-serum slants or slants of egg media can be carried out in a rice cooker. Place the tubes in the inner compartment of the cooker, obtaining the slant desired by manipulating an empty test tube, or with a towel or cotton batting on the bottom. Then cover the tubes with another towel. The outer compartment should contain water alone (not 25% salt solution). The inner compartment should be weighted down so that it is surrounded by water—the weight of the light tubes not being sufficient to sink it. With the water in the outer compartment boiling, one or two hours will suffice to inspissate or solidify the slants satisfactorily. The sterilization on subsequent days is best done in an Arnold or an autoclave, since the rice cooker, if used for this purpose, makes the media too dry.

Tellurite Medium for the Isolation of the Diphtheria Bacillus.—Nutrient or glucose agar is melted and cooled to  $50^{\circ}$ C. Add for each 10 cc. of agar 1 cc. of oxalated or defibrinated rabbit blood and 1 cc. of a sterile 2% solution of potassium tellurite. Mix and pour into Petri dishes. The covers should be tilted to dry off the water of condensation.

A Substitute for Ordinary Blood Serum.—Add from 10 to 15 cc. of 1% glucose broth to the white and yolk of one egg, make a smooth mixture in a mortar, and tube.

By flaming the mortar wet with alcohol, and by using a sterile knife for cracking the egg, it is possible so to reduce the bacterial content that sterilization of the medium can be accomplished as the result of the two-hour inspissation in the rice cooker. If these precautions are not taken, it will be necessary to sterilize after inspissation as for ordinary serum slants.

In morphology and luxuriance of growth, cultures of diphtheria bacilli on this medium are similar to those on Löffler's.

This medium seems to answer as a substitute for Dorsett's egg medium and is superior to the various white-of-egg substitutes usually recommended. When the medium is to be used for culturing tubercle bacilli, about r cc. of glycerin broth should be added to each tube before final sterilization in the autoclave. Although glycerin broth favors growth of human strains of tubercle bacilli, it is not so satisfactory for bovine strains as plain glucose broth.

Cultures of tubercle bacilli must be prevented from drying out by covering the tube with a rubber cap or by saturating the cotton stopper with melted paraffin or sterile vaseline.

Egg Media for Culturing Anaerobes.—The medium described above, with the addition of 1% neutral red solution in proportion of 5 drops to each egg, is of value in anaerobic work in that it makes colonies producing acid more distinctly visible.

Dorsett's Egg Medium.—This is prepared by breaking whole eggs into a sterile flask, mixing thoroughly, then adding 25 cc. water to every 4 eggs, straining through a sterile

cloth and tubing 10-cc. quantities. These tubes are slanted in an inspissator and kept at 73°C. for four or five hours on two successive days. On the third day a temperature of 76°C. is applied. Before inoculating add 3 or 4 drops of sterile water to each tube. Tuberculosis material should be rubbed into the surface well and the plugs paraffined.

Dorsett's Synthetic Medium for tubercle bacilli.—This medium, in which asparagin is substituted for complex protein substances, is used for the preparation of the purified protein derivative of tuberculin.

l-asparagin	14 Gm.
Dipotassium phosphate C.P	1.80 Gm.
Sodium citrate C.P	0 90 Gm.
Magnesium sulphate U.S.P.	1.50 Gm.
Ferric citrate U.S.P. scales	0.30 Gm.
Dextrose (cerelose)	10.00 Gm.
Glycerol C.P	100. CC.
Distilled water to	1000. CC.
Adjust to pH 7.0	

Lubenau's Egg Medium.—Put 10 fresh eggs in a 5 per cent solution of carbolic acid for 4 or 5 minutes. Wipe them dry with a sterile towel. Puncture both ends with a sharp pointed sterile instrument. Blow the contents of the egg through the larger end into a sterile flask. Add 200 cc. of a 5 per cent glycerin broth previously sterilized.

Mix thoroughly by shaking, strain through sterile cheese cloth, and fill into sterile test tubes by means of a sterile filling funnel. Place the tubes in a slanting position in the Arnold sterilizer. Bring the temperature slowly up to  $85^{\circ}$ C, allowing  $1^{\circ}$ L<sub>2</sub> hours to reach this temperature. Sterilize at  $85^{\circ}$ C, for 3 hours.

Petroff Medium.—Take 500 Gm. of round steak or veal, chopped fine, and 500 cc. of 15% glycerin in water. Mix and let stand in a cool place for 24 hours. Strain through cheese cloth. Mix 1 part of the glycerin meat juice and 2 parts of whole egg. To this add 1/10,000 parts of gentian violet. Mix and strain through cheese cloth, fill into tubes and place in Arnold sterilizer in a slanting position. Bring the temperature slowly up to 85°C. allowing 1½ hours to reach this temperature. On the second and third day heat to 75°C. for one hour each day.

Crystal Violet Potato (Corper).—Cut potatoes into cylinders 3 inches long and  $\frac{1}{2}$ 6 inch in diameter with a cork-borer and split them longitudinally. Soak for 2 hours in a freshly prepared solution of 1% anhydrous sodium carbonate containing 1-75,000 crystal violet. Wipe and place in sterile tubes containing 1.5 cc. of 5% glycerin broth. Sterilize at 15 lbs. for from 15 to 30 minutes.

Hydrocele, Serum, Ascitic and Milk Agar.—Melt tubes of 2 or 3% agar, cool to  $50^{\circ}$ C., and add from 1 to 3 cc. of sterile ascitic fluid or serum to each 10 cc. of agar. For milk agar we add the same quantity of plain or litmus milk. This makes an excellent plating medium for B. bulgaricus. The medium is opaque, but the colonies stand out well.

These fluids are obtained aseptically, and may be kept for a long time in the ice-box. Sterile serum must be removed from the clot before storing. It may be obtained from the arm vein in man, or from the heart in a rabbit. Slaughter house blood is less satisfactory for this purpose, but may be used. (See section on Löffler's medium.)

Blood Agar.—Sterile human blood is obtained from the arm vein, sheep blood from the jugular vein, or rabbit blood from the heart. Clotting is prevented by putting it into a flask of sterile 5% sodium citrate (1 cc. for each 10 cc. of blood), or by defibrinating it with sterile glass beads. The agar base is melted and cooled to 45°C. and the fresh blood is added in the proportion of from 5 to 10%. Pour immediately into Petri dishes or tubes. The growth is more easily seen if the blood agar is poured on plain agar slants and allowed to harden in a thin layer.

Whole Blood Agar for Differentiating Streptococci (Brown).— The base is 500 Gm. veal, 5 Gm. salt, 15 Gm. agar and 10 Gm. peptone per liter of media, prepared as for ordinary nutrient agar. The final reaction is from pH 7.4 to 7.6. The medium is tubed in 12-cc. amounts. To prepare blood agar melt down the base and place in water bath at 45°C. The tubes should be kept in the water bath about 15 minutes before adding 0.6 cc. of defibrinated blood and mixing thoroughly. The blood agar at 45°C is now inoculated with a loop or two of 24-hour culture of the streptococcus to be studied and then poured into Petri dishes to give a depth of 2 mm.

Oleate Haemoglobin Agar.—To make Avery's oleate haemoglobin agar add 5 cc. of 2.2% solution of neutral sodium oleate to 95 cc. of nutrient agar which should have pH of from 7.2 to 7.5. Outside of this range of reaction the Pfeiffer bacillus does not grow. While the oleate agar is still hot add defibrinated or citrated blood. Oleate blood agar is probably the most favorable medium for culturing the Pfeiffer bacillus, but cooked blood agar and ordinary blood agar are very satisfactory.

Chocolate (Cooked) Blood Agar.—Melt down nutrient agar, or preferably glycerin agar, and while the temperature of the medium in the tubes is about 90°C. add about 4 or 5% of citrated blood—0.5 cc. to 10 cc. agar in tube. Mix thoroughly, avoiding bubbles, and pour into plates. This cooked blood agar gives a luxuriant growth of Pfeiffer bacillus but is a dirty brownish opaque medium and is less satisfactory for isolating colonies than the ordinary blood agar.

Bordet-Gengou Medium for Isolating H. Pertussis.—Mix 500 grams of grated potato with 1000 cc. of distilled water containing 4% glycerin. Heat for an hour in the Arnold and squeeze through cheese-cloth. For each 100 cc. of potato extract add 200 cc. of 0.6% salt solution and 10 grams of agar. Sterilize in the Arnold for an hour on three successive days. For use, melt the agar, cool to 50°C. and add an equal quantity of defibrinated rabbit blood. This medium is used for the "cough plate" cultures.

Pure Blood Media.—For culturing the bacillus of chancroid (Ducrey) we use whole rabbit or human blood taken aseptically and preserved in small test tubes. The blood should be inactivated by heating the tubes for ½ hour at 56°C. This is a suitable medium for maintaining the virulence of pneumococci and streptococci.

In preparation of the agar base used for blood plates it has been found at the United States Naval Medical School that ordinary nutrient agar (2 to 3%) made up with Liebig's beef extract, salt, and peptone in the usual amounts gives satisfactory results. Many workers, however, insist on meat infusion agar; some laboratories have doubled the amount of peptone; some have omitted using salt, and some have added 1 to 2% glucose.

Avery's Medium for Grouping the Pneumococcus (artificial mouse).—Prepare a medium containing 18 parts of a meat infusion broth, 0.3 to 0.5 acid to phenolphthalein, sterilized without pressure; one part of a sterile 20% solution of dextrose, and one part of defibrinated rabbit blood. This should be tubed in 4-cc. quantities and not reheated.

Huntoon's Hormone Agar.-Take:

Beef heart, free from fat and finely chopped	500 Gm.
Whole egg	1
Peptone	το Gm.
Sodium chloride	5 Gm.
Agar, pulverized	18 Gm.
Tap water	1000 cc.

Mix in an enamelware dish and heat in a water bath (constantly stirring) until the color changes to brown—68 to 70°C. Titrate by adding 4 per cent NaOH until the medium reacts slightly alkaline to litmus paper. Add 1 cc. more of 4 per cent NaOH. Cover the vessel and place in an Arnold sterilizer for 1½ hours, or water bath at 100°C. Remove and separate the firm clot from the sides and return to Arnold sterilizer for 1½ hours. Remove and tip gently, allowing the fluid portion to accumulate at one side, which is now removed by means of a large pipette or siphon, avoiding any fat floating on the surface. Allow the agar to stand in a cylinder for 15 minutes. Skim off any fat present. Tube and sterilize in the usual manner.

If further clearing is desired, accomplish by sedimentation, filtration through glass wool, or centrifugation. The medium at no time must come in contact with cloth, cotton, or filter paper.

Plates made from this medium should be dried in the incubator for 1 hour before inoculation. Slant agar should be allowed to stand 24 hours before inoculation. For preserving stock cultures, the medium is the same as above, but only 5 grams of agar are used. Inoculate by stab method.

Fluid Medium.—Same as above, but substitute for agar 1 to 2 % gelatin.

Blood Glucose Cystine Agar (Francis).—This medium is used for original isolation of Bacterium Iularense and for subsequent cultivation. It is made as follows: Fresh beef infusion containing 1% peptone, r or 1.5% agar and 0.5% NaCl and adjusted to a pH of 7.3 is kept on hand in stock. When needed there is added to the stock agar 0.1% cystine (preferable) or cystine hydrochloride and 1% of glucose. This is placed at the temperature of flowing steam in an Arnold sterilizer sufficiently long to melt the agar and sterilize the cystine. The medium is now cooled to 60°C. and 5 to 8% of defibrinated or whole rabbit blood is added. Sterility is promoted by heating the flask of blood glucose cystine agar in a water bath for 2 hours at 60°C. avoiding a higher temperature which will cause sedimentation. The medium is then tubed from a sterile funnel and incubated to insure sterility.

This medium will also give abundant growths of the gonococcus and diphtheria bacillus.

## Selective Media for Gonococci

There has been a good deal of discussion of the importance of reduced oxygen tension in culturing gonococci. Investigators have suggested, however, that it is not so much the reduced tension that has been responsible for the good growths obtained as the fact that the methods employed to maintain this tension have insured the presence of an increased amount of moisture. McLeod et al. (1934) obtained a substantially higher percentage of growth in primary cultures incubated in an atmosphere of 8% CO<sub>2</sub> than in air.

Swartz's Medium.—This medium is made from a beef or veal infusion agar, prepared in the ordinary manner and brought to a reaction of pH 7.6. When the medium has

cooled to about 50°C., add the white of three fresh eggs to each 1000 cc. Starting with a low flame, boil for 10 minutes, strain through cloth and then filter through filter paper. Place 5 to 6 cc. of the medium in each test tube and autoclave at 10 pounds pressure on three successive days. This sterilization reduces the pH to 7.4. Now add sterile ascitic, pleuritic or hydrocele fluid to the melted agar in the proportion of one part of fluid to two parts of agar. The tubes are then sealed with sterile rubber stoppers and slanted. Corking, by preventing evaporation, permits storage of the media in the incubator, this, in turn, facilitating the detection of contamination and keeping the medium warm for inoculation at any time.

The medium is inoculated as richly as possible. It is important also to avoid letting the material cool pending inoculation and to have the medium at body temperature when inoculated.

Immediately after inoculation, the tube, held horizontal, is turned so that the agar slant is uppermost. Held by the butt, it is then passed longitudinally through the Bunsen flame about three or four times and quickly corked. Experiments with suitable apparatus show that this procedure heats the air in the test tube sufficiently to reduce the pressure within, when the tube has become cool, from 70 to 100 mm. of Hg (about 10% of atmospheric pressure) and, when properly carried out, does not coagulate the medium nor impair the viability of the culture. When the agar has set, the tubes should have about 0.5 cc. of water of condensation in the lower angle of the slant. The period of viability of the gonococcus on this medium is about seven days.

Pelouze and Viteri's Calf Brain Agar.—A calf's brain, weighing approximately 500 Gms. is forced through a wide-meshed gauze into 500 cc. of distilled water and placed in the ice-box for 24 hours. It is then filtered several times through cotton of varying degrees of compactness. The resultant fluid is turbid, no matter how often it is filtered. To this is added 0.5 per cent acid sodium phosphate and 1 per cent of peptone. It is then autoclaved at 15 pounds pressure for 20 minutes and kept as stock, or the final steps for its completion can be carried out.

To complete the medium it is necessary simply to add one part of the brain broth to three parts of standard 2.5 per cent agar medium made from veal broth, with the addition of 0.5 per cent of sodium chloride and one per cent of peptone. It should then be adjusted to a pH of 7.8 to allow for change, 7.6 being desired endpoint.

The medium is then tubed, autoclaved and slanted. After it solidifies the usual cotton plug is replaced by a sterile rubber stopper to retain the water of condensation and the medium keeps indefinitely.

Chocolate Blood Agar (McLeod et al., 1934).—Extract one pound of ground meat with a liter of 1% peptone solution containing 0.2% of Na<sub>2</sub>HPO<sub>4</sub> for 45 minutes at 60°C. and then for 30 minutes in an Arnold steamer. Adjust reaction to pH 7.4. Add the minimum amount of agar which gives a stable medium (about 1.3%). Sterilize and add 10% of heated blood.

Incubate the plates for 18 hours at 36°C. in a jar containing 8% CO<sub>2</sub> and then 24 hours in the air. Gonococcus forms convex, medium-sized, translucent colonies. Pour over the plate a sterile 1% solution of tetramethyl-p-phenylenediamine hydrochloride and drain off immediately. Gonococcus colonies quickly turn a bright purple color (oxidase reaction). Identification is completed by stains and subcultures (fermentation reactions and inability to grow on plain agar).

Kracke's Medium (for blood cultures).—The complement present in the blood is fixed, and immune bodies are rendered inactive by the minute particles of tissue in the

medium. The sodium citrate prevents clotting of the blood. The medium is kept in flasks in quantities of 50 cc. each and to this is added 10 to 15 cc. of blood. The formula is as follows.

Heart muscle extract	750 CC.
Brain suspension	250 CC.
Peptone	10 Gm.
Dextrose	
Sodium chloride	
Sodium citrate	
Dibasic sodium phosphate	

Heat until dissolved, adjust to pH  $_{7.4}$  and sterilize in the autoclave at  $_{15}$  lbs. for  $_{15}$  minutes.

Blood-streaked Agar.—Clean the lobe of the ear and puncture with a sterile needle. Collect the exuding blood on a large platinum loop and smear it over the surface of an agar slant. It is advisable to incubate over night as a test for sterility. In preparing this medium for culturing such organisms as pneumococci, streptococci, gonococci and meningococci, it is well to use glycerin agar, pH 7.6, as the base.

#### Bile Media

Secure ox bile from the abattoir or human bile from patients undergoing gall-bladder drainage in hospitals. Put about 10 cc. in each tube and sterilize. Some prefer to add 1% of peptone. Conradi's medium is ox bile containing 10% of glycerin and 2% of peptone. It is commonly used for blood cultures in suspected typhoid.

Bile lactose medium used in water analysis is made by adding 1% of lactose to ox bile and tubing in fermentation tubes. As a substitute for fresh bile one may use a 15 to 20% solution of a good quality of inspissated ox gall (Fel Bovis Purificatum). A liver broth made by using 500 grams of finely divided beef liver in 1000 cc. of water with 1% peptone, and prepared as for meat infusion broth, is a good substitute for bile.

## Plating Media for the Intestinal Bacteria

Of the numerous media proposed for plating out faeces, probably the most satisfactory are the Endo, Teague, bismuth sulphite and lactose litmus agar. In making any of these media, the following base is recommended in place of those advised in the original formulae. It is more easily prepared and yields equally good media.

Liebig's extract	5	Gm.
Salt	5	Gm.
Peptone	10	Gm.
Agar	30	Gm.
Water to make	T.000	cc

Prepare as for ordinary nutrient agar, adjusting the reaction to pH 7.8 to 8.2. A stiff agar (3%) is employed to check the diffusion of acid beyond the colony.

The "Standard Methods" Endo Medium.—This medium consists of a 3% agar, containing 5 Gm. of beef extract and 10 Gm. of peptone per 1000 cc. and adjusted to pH 7.8-8.2. It is flasked, sterilized and stored in quantities of 100 cc. When wanted, melt in streaming steam and proceed as follows, adding the ingredients in order given: To each 100 cc. add 5 cc. of a 20% solution of C.P. lactose sterilized by the fractional

#### CULTURE MEDIA

method, 0.5 cc. of stock basic fuchsin solution (10% alcoholic solution) and 0.125 Gm. anhydrous sodium sulphite dissolved in a small amount of hot distilled water. The sulphite solution must be made up fresh each time. Mix thoroughly. Pour plates at once and place in the incubator over night to harden and test sterility.

Colon bacilli show on this medium as vermilion colonies, which in about thirty-six hours have a metallic scum on them. Typhoid and dysentery colonies are grayish; streptococci, a deep red.

Lactose Litmus Agar.—Color roo cc. of neutral agar base to a lilac color with azolitmin, brom-cresol purple, or Andrade indicator. Then add 5 cc. of hot, freshly prepared 20% lactose solution in distilled water. This may be tubed, putting 10 cc. in each test tube, or put in quantities of 50 or 100 cc. in small Erlenmeyer flasks. It is then sterilized in the autoclave at 10 pounds for fifteen minutes, or in the Arnold.

Teague Medium.—We have obtained most satisfactory results with the Teague medium. On this, colon colonies, after eighteen hours, are deep-black and opaque whereas those of the typhoid-dysentery group are colorless and transparent. After thirty-six hours the plates are not very satisfactory.

The medium is prepared as follows: Nutrient agar is made in the usual way, containing 1.5% agar, 1% peptone, 0.5% sodium chloride, and 0.5% Liebig's meat extract, to the liter of distilled water. It is cleared with egg-white, placed in flasks, and sterilized in the Arnold sterilizer on three successive days. The reaction is brought to pH 7.4. The agar is melted, and saccharose 0.5% and lactose 0.5% are added. The medium is then heated for ten minutes in the Arnold. To every 50 cc. of the medium are added first, 1 cc. of 2% yellowish eosin and then 1 cc. of 0.5% methylene blue. The mixture is shaken and plates poured.

Bismuth Sulphite Medium (Wilson and Blair).—To 100 cc. of the above agar base add the following. Five cc. of a 20% solution of glucose; 10 cc. of a 20% solution of anhydrous sodium sulphite solution; and 5 cc. of a standard bismuth solution. Boil 2 minutes and add 1 Gm. anhydrous sodium phosphate and 1 cc. of an 8% solution of ferrous sulphate.

The standard bismuth solution is prepared by adding to 60 Gm. of bismuth citrate 50 cc. of distilled water and 20 cc. of ammonium hydroxide (sp. gr. 0.880) bringing the volume up to 500 cc. with distilled water.

Brilliant green (0.5 cc. of a 1% aqueous solution) may be added if desired.

On this medium the colonies of *E. typhosa* are black due to the ability of the organism to reduce the sulphite to sulphide in the presence of glucose. The growth of *E. coli* is inhibited.

Eosin Methylene-blue Agar.—Add 10 Gm. of Difco peptone, 2 Gm. of dipotassium phosphate  $(K_2HPO_4)$  and 15 Gm. of undried agar to 1000 cc. of distilled water. Boil until all ingredients are dissolved and make up any loss due to evaporation with distilled water. Adjustment of reaction is not necessary. Place measured quantities (100 or 200 cc.) in flasks or bottles and sterilize in the autoclave as directed at 15 lbs. for 15 minutes.

Just prior to using, melt stock agar and add the following ingredients to each 100 cc.: Lactose, sterile 20% solution, 5 cc. Eosin, yellowish, 2% aqueous solution, 2 cc. Methylene-blue, 0.5% aqueous solution, 2 cc. Mix thoroughly, pour into Petri dishes, allow to harden and inoculate by streaking on the surface.

It is permissible to add all the ingredients to the stock agar at the time of preparation, place in tubes or flasks and sterilize. Decolorization of the medium occurs during sterilization. The color returns after cooling. Desoxycholate Agar (Leifson).—In 1000 cc. of distilled water dissolve 10 Gm. of peptone, bring to a pH of 7.3 to 7.5, boil a few minutes and filter. Add agar, 12 Gm. (for poured plates) to 17 Gm. (streaked plates), soak 15 minutes or more, melt, and add 6 cc. of N NaOH. Then add rapidly in order, sodium chloride, 5 Gm.; lactose, 10 Gm.; ferric ammonium citrate, green scales, 2 Gm.; dipotassium phosphate, 2 Gm.; sodium desoxycholate, 1 Gm. Bring to a pH of 7.3 to 7.5 (using phenol red) and add 3 cc. of a 1% aqueous solution of neutral red. Sterilize in an Arnold steamer just long enough to kill vegetative cells; 15 minutes for tubes, longer for flasks. The peptone must be suitable. Leifson recommends proteose or Fairchild's for general purposes.

Escherichia and practically all the intestinal pathogens grow well, including the dysentery bacilli. Cocci and Gram-positive bacilli are inhibited. *Proteus* colonies do not spread.

Desoxycholate-citrate Agar (Leifson).—Infuse 333 Gm. of fresh, finely ground, lean pork for one hour in 1000 cc. of water, add 3.3 cc. of N HCl, boil one minute, strain and filter until clear. Add 3.3 cc. N NaOH, boil one minute, filter and restore volume to 1000 cc. Add 10 Gm. of peptone (proteose, bacto, or Fairchild's are satisfactory), and bring to a pH of 7.5. Boil 2 to 3 minutes and filter. Add 20 Gm. agar, soak 15 minutes or longer, melt, and quickly add in order: lactose, 10 Gm.; sodium citrate (2Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7.11</sub>H<sub>2</sub>O), 25 Gm.; sodium desoxycholate, 5 Gm., and (optional) lead chloride, 3.5 mg. Bring to a pH of 7.4 (with phenol red), and adjust volume. At this stage the medium may be stored in sterile bottles.

Just before use add to the hot melted agar 2 Gm. of ferric ammonium citrate in neutralized solution and 2 cc. of a 1% solution of neutral red. Pour into Petri dishes without further sterilization. Overheating must be avoided. For important details consult original paper (J. Path. and Bacteriol., 1935, XL, 581).

In addition to cocci and Gram-positive bacilli, this medium inhibits the growth of most strains of colon bacilli, Alcaligines, some strains of Salmonella, and Shiga (stock strains), Sonne, alkalescens and dispar types of dysentery bacilli. Typhoid, both paratyphoids, suipestifer, the Schmitz and Flexner group of dysentery bacilli, and others grow well. Proteus colonies do not spread (they lose their flagella while grown on this medium). It inhibits the growth of the normal intestinal flora so strongly that large amounts of faeces can be streaked on this medium.

Russell's Double Sugar Agar.—To a fairly stiff agar (2 to 3%), with a reaction of about pH 7.4, is added enough azolitmin solution to produce a distinct purple-violet color or, preferably, Audrade's indicator. It may be necessary to add more alkali. To this litmus-tinted agar is added  $t \, C_0$  of lactose and  $o.t \, C_0$  of glucose, and the medium as thus prepared is tubed and slanted. Sterilization should be carried on in the Arnold, on two successive days, as the autoclave temperatures tend to break up the sugars.

To inoculate this medium take material from a suspected colony grown on Endo and smear the material on the slant; then with the same platinum needle stab into the butt.

On these litmus slants typhoid shows a delicate growth on the violet slant with a deep pink in the butt of the tube. The paratyphoids show gas bubbles in a pink butt with a violet slant. The colon bacillus turns both slant and butt a deep pink, and the butt is filled with gas bubbles. With Andrade's indicator the acidified areas are pink and the rest is colorless.

Lead Acetate Medium.—To make this medium, take 1.5% nutrient agar and add 1% glucose, 1% lactose and 0.05% basic lead acetate. The basic lead acetate solution is made up as a 0.5% solution and sterilized and the requisite amount added to each

tube before slanting. The same technique is followed with this medium as with Russell's, stabbing into the butt of the tube and streaking the slant.

This medium is useful in differentiating the paratyphoids. Paratyphoid B organisms give a brownish discoloration which is not given by typhoid or paratyphoid A. The gas production is similar to that with the Russell tube.

Tartrate Medium (Jordan).—Dissolve agar, 20 Gm.; peptone (Difco), 10 Gm.; sodium potassium tartrate, 10 Gm.; and NaCl, 5 Gm. in 1000 cc. of distilled water. Adjust the reaction to a pH of 7.4, and add 12 cc. of a 0.2% alcoholic solution of phenol red. Tube, sterilize in the autoclave at 15 pounds pressure for 15 minutes, and cool in upright position. Inoculate by stab. Check medium by inoculation with stock cultures. S. enteritidis, S. aertrycke, and S. suipestifer should form acid, the paratyphoids should not.

## Selective Media for Cholera

Dieudonne's Medium.—The value of Dieudonne's medium depends upon the ability of cholera to grow when alkali is present in such amounts as to inhibit the growth of other faecal bacteria.

Take equal parts of defibrinated blood obtained at the slaughter house and normal NaOH solution. Mix 30 parts of this alkaline blood mixture with 70 parts of hot 3% nutrient agar. The poured plates must be left half open over night in the incubator to dry, otherwise even cholera will not grow on them.

Krumwiede's Medium.—Krumwiede's formula is as follows. Take equal parts of whole egg and water, and add to the mixture an equal volume of 12.5% sodium carbonate (crystals) solution. Having steamed this alkaline egg mixture for twenty minutes, add 30 parts to 70 parts of meat extract-free 3% agar. (No meat extract; only peptone and salt.) The surface of the agar must be dry. The cholera colony has a hazy look, like a little wad of absorbent cotton sticking to the surface, with a metallic-luster halo.

Goldberger's Medium.—First prepare a meat infusion by treating 500 grams of finely chopped lean beef with 500 cc. water. After three hours strain the infusion, adjust reaction to neutral with 5.3% anhydrous sodium carbonate, then add to each 100 cc., 2.5 cc. of 5.3% anhydrous sodium carbonate solution, sterilize in Arnold for one-half hour and filter. Next prepare a 3% meat extract agar and mix one volume of the alkaline meat infusion with 3 volumes of the hot melted 3% meat extract agar. Pour plates and cover with a piece of filter paper and place in incubator for one-half hour until they are quite dry. On this medium cholera grows well while faecal bacteria are restrained. The cholera colony is clear, round, and shows a brownish center but is without that striking bluish opalescence shown on ordinary agar plates.

Esch Medium.—This medium has been highly recommended. It is easy to make. Heat 500 grams chopped-up beef with 250 cc. normal NaOH solution in a pot and when disintegrated filter through cloth and sterilize. About 1 part of this alkaline extract is added to  $2\frac{1}{2}$  to 2 parts of nutrient agar. The plates must be dry. The transparency of this medium is an advantage.

Aronson's Medium.—This is an excellent medium for the examination of stools of cholera carriers. The organisms taken from such plates emulsify easily and there is no interference with their agglutinability. To prepare it add to 100 cc. of 3% nutrient agar, 6 cc. of 10% solution of exsiccated sodium carbonate and steam in Arnold sterilizer for fifteen minutes. Then add 5 cc. of 20% saccharose solution, 5 cc. of 20% dextrin solution, 0.4 cc. saturated alcoholic basic fuchsin and 2 cc. of 10% sodium sulphite.

## MEDIA FOR SPIROCHAETES

A precipitate forms which quickly settles and plates can be poured from the supernatant fluid. Cholera colonies develop in twelve hours and show as red colonies in fifteen to twenty hours. Colon colonies are much larger than these and are colorless. In stock cultures of the cholera vibrio the colonies are much slower in development.

## Media for Spirochaetes

Noguchi's Medium (for Treponemata).—Noguchi formerly first inoculated material containing treponemata into the testicle of rabbits, obtaining by this procedure a pure culture, after a few transfers to the testicles of other rabbits. Later he was able to grow the organism directly from serum from a chancre. Test tubes 2 by 20 cm. are filled with 15 cc. of a medium consisting of 2 parts of 2% slightly alkaline agar (pH 6.5 to 7.0) to which when melted and cooled down to 50°C. is added 1 part of ascitic or hydrocele fluid. At the bottom of the medium in the tube is placed a fragment of fresh sterile tissue, preferably a piece of rabbit's kidney or testicle. After the medium solidifies a layer of sterile paraffln oil is run in so that it covers the solid medium to a depth of 3 cm. in order to prevent evaporation. The material is inoculated at the bottom of the tube with a capillary pipette. Incubation at 37°C. is carried on for two weeks under anaerobic conditions. The tissue acts by removing any oxygen that may be present in the depths of the medium, anaerobiosis being a necessary condition. obtain anaerobic conditions Noguchi uses an anaerobic jar in which these conditions are produced by a combination of vacuum, hydrogen gas and pyrogallic acid. It is to be noted that many specimens of ascitic fluid are unsuitable. The tubes of Noguchi and Bronfenbrenner are shown in Fig. 203. Bronfenbrenner uses a 1.5% agar instead of the 2% used by Noguchi.

 $M^{\prime}Lcod$  and Soga have simplified Noguchi's procedure as follows: Take a test tube and fit a perforated rubber stopper which can be pushed down the tube. A piece of glass tubing is passed through the stopper to project slightly into the test tube. The other end of the glass tube is drawn out into a capillary tube and bent over at an acute angle. The test tube is filled to  $\frac{1}{2}$  or  $\frac{2}{3}$  of its depth with neutral broth. This is freshly boiled and when cool a piece of sterile tissue is dropped in. A strip of sterile gauze is drawn through a glass bead and soaked in the material it is desired to culture and dropped into the bottom of the tube alongside the fragment of sterile tissue. Ascitic fluid is then run in to a point which would be reached by the bottom of the rubber stopper. As quickly as possible push in the stopper and when the fluid appears in the capillary tube scal off the end in a small flame. Material for study can be obtained afterwards by breaking off the capillary tip and introducing a capillary pipette.

Noguchi's Medium (for Leptospira).—This consists of one part of rabbit serum and three parts of Ringer's solution made semisolid with 0.3% agar and contained in tall tubes. One cc. citrated blood is introduced into the lower part of the medium. A thin layer of liquid petrolatum is poured on the top of the medium.

Fletcher's Medium for Leptospira.—Heat to 50°C. a 12% solution of rabbit serum in distilled water. Add 6 cc. of 2.5% agar to each 100 cc. of serum-water mixture. Adjust reaction to a pH of 7.4. Tube in 5 cc. quantities and sterilize at 56°C. for one hour on two consecutive days. Inoculate with 0.1 cc. of infected guinea pig blood or liver suspension or rat kidney suspension. Incubate aerobically at 25° to 30°C. Subculture every 4 to 6 weeks.

For the cultivation of the Borrelia group Noguchi recommends ascitic fluid (without agar) plus fresh tissue, the selection of a suitable sample of ascitic fluid being an

important factor. A suitable ascitic fluid causes a loose fibrin formation in the culture tube when mixed with the fresh tissue. The medium is covered with a thin layer of paraffin oil to hinder evaporation.

# Culture Media for Filtrable Viruses

The following methods are suitable for the multiplication of a number of the filtrable viruses.

Maitland and Maitland Tissue Medium.—This consists of fresh, minced adult rabbit (or hen) kidney (or testicle) and sterile filtered Tyrode's solution. (Their first medium called for fresh serum in addition, but this was found to be unnecessary.) The kidney is removed aseptically and added to the Tyrode's solution after it is minced in a proportion of about 0.3 Gm. to 10 cc. The virus may be added during the mincing. The mixture is placed in Erlenmeyer flasks in a thin layer—about 2 mm. thick. One kidney will make a number of flasks of the medium. The tissue cells remain alive for about five days at which time multiplication of the virus has usually reached its maximum. Some viruses require a particular type of tissue.

Chick Embryo-Tyrode's Solution (Rivers).—This medium is prepared with minced embryos from eggs incubated from 9 to 12 days. The eggs are opened aseptically, and the embryos cut into bits with sterile scissors. Sterile filtered Tyrode's solution is added in the proportion of about 10 cc. for each 0.2 Gm. of minced embryo. This is distributed in Pyrex Erlenmeyer (or special collar) flasks in a thin layer, and the cotton stopper is covered with tin foil. The inoculated flasks are incubated at 37°C. for 5 days.

Tyrode's solution consists of—NaCl, 8 Gm.; KCl, o.2 Gm.; CaCl<sub>2</sub>, o.2 Gm.; MgCl<sub>2</sub>, o.1 Gm.; NaH<sub>2</sub>PO<sub>4</sub>, o.05 Gm.; NaHCO<sub>3</sub>, 1 Gm., glucose, 1 Gm., distilled water to 1000 cc. The living tissue corrects the pH of the solution. Sterilize by filtration.

In all tissue cultures extreme care must be used to avoid bacterial contamination, and each culture should be checked by smears and culture for bacteria before making subcultures.

## Cultivation of Fungi

Moulds grow well on media with an acid reaction, so that by adjusting the reaction to pH 5.5 to 6.0 or even lower, we permit the growth of the fungi, but inhibit bacterial development. Glycerin agar, bread paste, or potato media are all suitable, but the best media are those of Sabouraud:

Sabouraud's Medium for Fungi.—Conservation medium (for preserving stock cultures).

Peptone	30.0 Gm.
.\gar (shred)	15.0 Gm.
Tap water	1000.0 Gm.

Differentiation media (two of these are used—a maltose medium and a glucose medium).

Maltose (or glucose)	40.0 Gm.
Peptone	10.0 Gm.
Agar (shred)	15.0 Gm.
Tap water	Tooo o Gm

In each case the ingredients are added to the water and all placed in a cold autoclave and the pressure allowed to rise in both outer and inner jacket simultaneously until it has reached 15 pounds. The autoclave is then shut off and allowed to cool down slowly. When autoclave has cooled, the medium is filtered through cotton, tubed, and then sterilized the same as above. Upon removal from the autoclave, the tubes are slanted and allowed to cool in this slanting position. No adjustment of the pH is necessary.

# Culture Media for Intestional Protozoa

Boeck and Drbohlav's Media.—E. histolytica and intestinal flagellates have been successfully cultivated on the following media.

(1) Locke egg-scrum or L.E.S. medium which is prepared as follows.

Four eggs are washed, brushed with alcohol and broken into a sterile flask containing glass beads. Fifty cc. of Locke's physiological solution are added and the mixture broken up by shaking. Test tubes are then filled with a sufficient quantity to produce slants from about 1 to  $1^{1/2}$  inches upon coagulation by heat. These tubes are now slanted in an inspissator and heated (70°C.) until the egg mixture has solidified. They are then transferred to the autoclave and sterilized for 20 minutes at 15 pounds pressure.

The tubes are now covered to a depth of 1 cm, above the egg slant with a mixture composed of 8 parts of sterile Locke's solution and one part of sterile inactivated human blood scrum. They are then incubated to determine sterility.

## LOCKE'S SOLUTION

Distilled water	1000.00	cc.
NaCl	9.0	Gm.
$CaCl_2$	0.2	Gm.
KCl	0.4	Gm.
$NaHCO_3$	0.2	Gm.
Glucose.	2.5	Gm.

(2) Locke egg-albumin or L.E.A. medium which is prepared by covering the egg slants with Locke's solution containing 1% of crystallized egg albumin. It has the advantage over the L.E.S. medium of being more readily prepared since the albumin is usually more available than human serum.

The best growth of amoebae occurs between pH 7.2 and 7.8 which is usually the pH of the L.E.S. and L.E.A. media. Adjustment, however, may be required.

Cleveland and Collier's medium (1930).—(1) Liver infusion agar (Difco dehydrated) 30 Gm. (2) Disodium phosphate 2 Gm. (3) Distilled water 1000 cc. Autoclave and slant. Cover the slants with a 1 in 6 dilution of sterile fresh horse serum in physiological salt solution and add a 5 mm. loop of sterile rice flour or powdered unpolished rice. In making subcultures remove two or three drops of the rice flour debris from the bottom with a sterile, wide-mouth, glass pipette. These authors reported obtaining E. histolytica in nearly every case, despite bacterial contamination.

Barret and Yarbrough's Medium.—Barret and Yarbrough have succeeded in culturing Balantidium coli in vitro through eleven transplants over a period of 32 days. They used a medium composed of inactivated human blood serum and 0.5% salt solution in the proportion of one part of serum to sixteen parts of salt solution. This medium is faintly alkaline to litmus. Eight-cc. quantities of medium were placed in

tubes having a diameter of 10 mm. and a length of 150 mm., giving the medium a depth of about 100 mm. Inoculation of about 0.1 cc. of undiluted faeces containing mucus was made with a capillary pipette into the bottom of the tubes in order to insure the partial anaerobic conditions which favor the growth of the balantidia. The tubes were then incubated at 37°C. and subcultures were in general made every second day. Subsequent examinations showed that active balantidia were present only in the lower portion of the tube and that a moderate growth of bacteria as shown by a moderate clouding of the medium seemed to favor the balantidial growth.

#### Culture Media for Other Protozoa

N.N.N. Medium (Nicolle Novy MacNeal).—This is used extensively for the cultivation of trypanosomes and *Leishmania*. With human trypanosomes, rat or human blood is substituted for the rabbit blood specified.

Take 14 Gm. agar, 6 Gm. salt and 900 cc. water. Prepare as for ordinary agar, tube and sterilize. To 1 part of this medium liquefied and cooled to 48°C., add one-third its volume of defibrinated rabbit's blood. Mix thoroughly, slant, and allow to set. Use rubber stoppers or cover cotton plugs with melted paraffin to prevent evaporation of the condensation fluid. It is the Hb. which seems essential in cultivating various blood protozoa. Inoculate the water of condensation with the suspected material and incubate for several days at 22° to 25°C.

Row's Medium for Leishmania.—Take 10 cc. blood from rabbit's heart or arm vein of man, defibrinate the blood and then add 10 volumes of distilled water to lake the cells (liberation of Hb.). One volume of this laked blood solution is added to two volumes of sterile 1.2% salt solution.

Rogers used as a medium for Leishmania blood containing a small quantity of sterile 10% sodium citrate solution slightly acidified with citric acid. One or two cc. of the citrate solution were placed in the barrel of a syringe into which the splenic blood was aspirated directly. Incubation at 22°C. seemed essential to the development of the flagellated forms, higher temperatures shortening the period of growth or causing disintegration of the organisms. Other observers, having noted that certain organisms of this group develop well at 28°C., have used this characteristic as a basis for species differentiation.

Bass' Medium.—For the cultivation of malarial organisms, Bass takes from 10 to 20 cc. of blood from the malarial patient's vein in a centrifuge tube which contains 0.1 cc. of 50% glucose solution. A glass rod, or a piece of tubing, extending to the bottom of the centrifuge tube is used to defibrinate the blood. After centrifugalizing there should be at least one inch of serum above the cell sediment. The parasites develop in the upper cell layer, about  $\frac{1}{50}$  to  $\frac{1}{20}$  inch from the top. All of the parasites contained in the deeper-lying red cells die. To observe the development, red cells from this upper  $\frac{1}{20}$ -inch portion are drawn up with a capillary bulb pipette.

Should the cultivation of more than one generation be desired, the leukocyte (upper) layer must be carefully pipetted off, as the leukocytes immediately destroy the merozoites. Only the parasites within red cells escape phagocytosis. Sexual parasites are much more resistant. Bass thinks he observed parthenogenesis. The temperature should be from 40° to 41°C.; and strict anaerobic conditions observed. Aestivoautumnal organisms are more resistant than benign tertian ones. Dextrose seems to be an essential for the development of the parasites.

# C. STAINING METHODS

### I. For Bacteria

The morphology of the different bacteria is studied chiefly by means of stained smears, and the appearance of certain species of bacteria stained by special methods may be sufficiently characteristic to identify them. In order to study the activities of living bacteria, however, it is necessary to observe them in a hanging drop.

Hanging-drop Preparations.—To prepare a hanging drop place a loopful of the young broth culture, (or a loopful of salt solution into which is then emulsified a small amount of growth from an agar slant) in the center of the cover glass; now having applied with a brush a ring of vaseline around the concave depression in the slide, apply the slide as a cover to the cover glass which latter adheres to the ring of vaseline. The completed hanging-drop preparation can now be turned over and placed on the stage of the microscope.

An alternative method, equally good, consists in spreading a ring or square of vascline—smaller than the cover glass to be used—in the middle of a plain slide. Then put a loopful of salt solution in the center of the space, inoculate with the culture to be studied, and finally cover it with a cover glass, gently pressing the margins down on the vaseline. This gives a preparation for the study of motility or agglutination which does not dry out for hours, and is easier to focus upon than the concave slide hanging-drop preparation.

In examining a hanging drop first use a low-power objective and, having brought into focus the margin of the drop as a center line, change to a ½- or ½-inch objective. By this procedure a thin layer of fluid is brought under the high dry objective instead of the deeper layer in the center of the drop. It is not advisable to use an immersion objective with a hanging-drop preparation owing to great difficulty in focusing and the possible detachment of the cover glass by sticking to the oil objective. The light should be cut down to a minimum with the iris diaphragm and the concave mirror used. A dark-ground illumination is necessary for observing spirochaetes.

Hanging-drop preparations can be used to observe the growth of bacteria if the slides and cover slips are sterile and the preparation is well sealed. A small block of agar (or any transparent solid medium) may be used in place of the broth. A thin square is cut out and placed on a sterile slide. The upper surface is inoculated, and a sterile cover glass is placed over it. The cover glass and agar block are then removed from the slide and placed over a vaseline-ringed hollow-ground glass slide.

Intravital Staining.—A perfectly clean slide is flooded with a saturated aqueous solution of methylene blue or 10% toluidin blue to form an even film. When dry the slide has a clean blue color. To stain organisms deposit a loop of the culture, or material emulsified in saline, on a cover glass. Mount on the stained slide. The organisms take up the stain.

Stained Preparations.—To make a preparation, apply a very small loopful of distilled water on a slide or cover glass (cleaned as described in section A) and, touching a colony with a platinum needle, stir the transferred culture into the loopful (not drop) of water. The mistake of taking up too much bacterial growth is almost invariably made. Fluid cultures do not need dilution. The mixture is smeared over a large part of the cover glass, or over an equal area of a slide, and it is allowed to dry. If very little water is used, the preparation dries readily. Otherwise it can be dried in the fingers

high over a flame. As soon as dry, the cover glass should be passed three times through the flame, film side up, to fix the preparation. Slides also may be fixed by passing them five times through the flame, but the method by burning alcohol recommended for fixing blood films gives more satisfactory bacterial fixation. For routine work the stain recommended is a dilute carbol fuchsin (1–10) or Löffler's methylene blue. Drop about 5 to 10 drops of water on the cover glass, then add 1 drop of carbol fuchsin. Allow the dilute stain to act from one to two minutes, then wash in water, dry between small squares of filter paper, and mount in balsam or in the oil used for the  $\frac{1}{12}$ -inch immersion objective. As a rule, better definition is obtained with dilute stains than with more concentrated ones.

If, in the study of cells, granular differentiation is wanted, fixation with alcohol, osmic acid, etc., should be used. If only nuclei and cell outlines are desired, so that intracellular bacteria may be distinct against a perfectly clear back ground, fixation with alcohol and immediate flaming is preferable. If flamed at once the cytoplasm of the cells does not take the stain, and the nucleus and bacteria appear to be enclosed in a clear ring. The longer the alcohol is left in contact with the smear the more completely will the cytoplasm be stained.

Liquid petrolatum is by far the best mounting medium. It is always of proper consistence and is never sticky. Unlike Canada balsam, it does not develop any acidity and permanent mounts retain their color for at least 2 years. This is particularly important with preparations of blood, malarial parasites, trypanosomes, intestinal

Per cent soluble in

Name of dye	Water	95 per cent alcohol
Bismarck brown Y	1.36	80.1
Crystal Violet (chloride)	• 1.68	13.87
Eosin Y (Na salt)	44.20	2.18
Erythrosin (Na salt)	II.IQ	1.87
Fuchsin (basic) Rosaniline (chloride)	0.39	8.16
Para Rosaniline (chloride)	0.26	5.93
Para Rosaniline (acetate)	4.15	13.63
New fuchsin (chloride)	1.13	3.20
Janus green	5.18	1.12
Malachite green (oxalate)	7.60	7 . 5 2
Methyl violet	2.93	15.21
Methylene blue (chloride)	3.55	1.48
Neutral red (chloride)	5.64	2.45
Picric acid	1.18	8,96
Pyronin B (iodide)	0.07	1.08
Pyronin G	8.96	0 60
Safranin	5 · 45	3.41
Thionin	0.25	0.25
Toluidine blue (O)	3.82	0.57

flagellates, etc., in which a well stained chromatin is necessary for identification. It is also useful for mounting small insects and sporangia of moulds. Furthermore it has superior optical properties. For permanent preparations the border of the cover glass should be sealed with gold size or some other cement. Some prefer to mount directly in water without preliminary drying. In grasping a cover glass with a Cornet or Stewart forceps, be sure that the tips are well inside the margin of the glass, otherwise the stain will drain off.

It is convenient to keep on hand saturated alcoholic solutions of the various dyes from which to prepare the ordinary bacterial stains. The list shown in the table on p. 858 indicates the quantities of the different dyes required to obtain saturated solutions.

## STAINS FOR GENERAL USE

Löffler's Alkaline Methylene Blue.—Saturated alcoholic solution of methylene blue, 30 cc.; I to 10,000 caustic potash solution, 100 cc. (Two drops, or  $\frac{1}{10}$  cc., of a 10% solution KOH in 100 cc. of water makes a 1:10,000 solution.)

Carbol Fuchsin (Ziehl-Neelsen).—Saturated alcoholic solution basic fuchsin, 10 cc.; 5% aqueous solution carbolic acid, 100 cc. This solution, diluted 1-10, makes a good stain for general use. The diluted stain does not keep well, however, and must be prepared frequently. The dilution can be made directly on the slide by dropping on it 9 drops of water and 1 of the concentrated stain.

**Gram's Method.**—This is the most important staining method in bacteriological technique. It is, however, likely to give unsatisfactory results in the hands of the inexperienced and in using it the following points must be kept in mind:

Laboratory cultures (subcultures) which have been carried over for years frequently lose their Gram characteristics. Cultures which are several days old or dead or degenerated do not stain characteristically. The iodine solution deteriorates and becomes light in color. It should be of a rich port-wine color. Decolorization, best observed over a white background, should be stopped as soon as no more violet stain is seen to stream out from the preparation. Decolorization can be controlled by making a preparation of a known Gram-positive and a known Gram-negative organism on either side of the snear to be stained. The preparation should be thin and evenly spread.

Stains and other reagents used:

- 1. Gentian violet.—(a) The original stain recommended was aniline gentian violet. This is made by mixing 1 part of saturated alcoholic solution of gentian violet with 3 parts of aniline oil water, which is prepared by adding 2 cc. aniline oil to 100 cc. distilled water, shaking violently for 3 to 5 minutes and then filtering several times to get rid of the objectionable oil droplets which, in a Gram-stained preparation, show as confusing black dots. This stain does not keep well and is seldom used.
  - (b) Weigert has recommended the following stock solution:

No. 1.		No. 2	
Gentian violet			
Aniline oil	9 cc.	Distilled water	100 CC.
Alcohol (95%)	33 cc.		

These solutions keep indefinitely. To prepare the solution used in staining, mix I cc. of No. I with 9 cc. of No. 2 and filter. This keeps about two weeks.

(c) Carbol gentian violet is preferred by some to aniline gentian violet. It is made by adding r part of saturated alcoholic solution of gentian violet to 10 parts of 5%

aqueous solution of carbolic acid. This has a tendency to overstain. Crystal violet can be substituted for the gentian violet in any of these formulae and is more satisfactory.

Ammonium Oxalate Crystal Violet.—Saturated alcoholic solution of crystal violet, 25 cc.; 1% aqueous solution of ammonium oxalate, 100 cc. If this solution stains too deeply the proportion of the crystal violet should be reduced. This stain keeps indefinitely.

Kopeloff and Beerman Modification.—One per cent aqueous solution of crystal or methyl violet, 30 parts; five per cent solution of sodium bicarbonate, 8 parts. Mix just before using. The iodine solution used in this method is alkalinized. The formula is, iodine 2 Gm., normal solution of sodium hydroxide, 10 cc., distilled water 90 cc. Acetone is recommended for decolorization. The technique of staining is similar otherwise to that of the other methods.

2. Gram's iodine solution.—The formula for this solution is:

Iodine	ı Gm.
Potassium iodide	2 Gm.
Distilled water	300 cc.

Sheppe and Constable demonstrated that when exposed to light and heat the iodine solution used in the Gram stain may become acid, owing to the formation of small amounts of hydriodic acid, which exercised a decolorizing effect on normally Gram-positive bacteria. According to Kilduffe, sodium bicarbonate will neutralize the acid formed in the iodine solution as well as any that may be present in smears made from acid secretions.

His formula for the iodine solution is:

Iodine	т Gm.
Potassium iodide	2 Gm.
Distilled water.	240 CC.

When dissolved, add 60 cc. of 5% aqueous solution of sodium bicarbonate.

3. Decolorizing agents.—Alcohol 95% was originally recommended as the decolorizing agent, but acetone is preferred by many owing to the fact that it is more rapid in its decolorizing action on Gram-negative organisms, yet slower to decolorize positive organisms. Acetone gives also a cleaner preparation than alcohol, particularly in smears of pus and sputum.

Counterstains.—The following stains are most commonly recommended.

Safranin, made by mixing 10 cc. of a saturated alcoholic solution of water-soluble safranin with 90 cc. of distilled water. This stain keeps.

Dilute carbol fuchsin, made by mixing 1 part of carbol fuchsin with 10 parts of water. Bismarck brown. This is prepared by dissolving 0.2 Gm. of the stain in 100 cc. of boiling water. It is then cooled and filtered.

Technique of Gram staining:

The preparation to be stained should be thin and evenly spread. The film should be air-dried and passed through flame for fixing. The gentian violet is then applied and kept on for 2 to 5 minutes. Some hasten the staining by steaming as for tubercle bacilli. Next wash the preparation with water and then flood it with Gram's iodine solution. Some bacteriologists simply pour off excess of aniline gentian violet and immediately drop on the iodine solution. It is well to repeat the application of the

iodine solution a second time. The iodine solution is left on one minute or until the preparation has the color of coffee grounds.

Wash off the excess of iodine solution at the tap, drop on 95% alcohol, or acetone, and decolorize until no more violet color streams out. Now wash again and counterstain with one of the stains mentioned above.

Gram-positive bacteria are stained a deep violet whereas the Gram-negative organisms take the color of the counterstain.

In staining smears of pus for gonococci or other Gram-negative bacteria it is best first to stain with the gentian-violet solution for two to five minutes. Then wash and examine the preparation mounted in water. The organisms stand out prominently. After noting the presence of the cocci treat the smear with the iodine solution and proceed as in the usual Gram-staining technique.

# Gram Staining Reactions of Important Bacteria Gram Negative Gram Positiv

Gram Negative	Gram Positive
Cocci	Cocci
Meningococcus	Staphylococcus group
Catarrhalis	Streptococcus group
Gonococcus	Pneumococcus group
	G. tetragena
Bacilli	Bacilli
K. pneumoniae	C. diphtheriae group
P. aeruginosa	C. tetani
F. dentium	M. tuberculosis
E. coli group	M. leprac
E. typhosus group	B. anthracis
S. dysenteriae group	C. welchii and other anaerobes
Λ. mallei	
C. chauvoei	
P. tularensis	Spirilla
P. pestis	None important
H. influenzae	
H. pertussis	
H. ducreyi	
H. lacunatus	
H. conjuncturitidis	
Brucella group	
Spirilla	Gram amphophil (variable)
Cholera and allied forms	Molds
Mouth spirals	Yeasts

Methods for Staining Acid-fast Bacilli. Ziehl-Neelsen's Method.—1. Apply carbol fuchsin, steam gently for three to five minutes, or stain in the cold for fifteen minutes. 2. Wash in water. 3. Decolorize in 95% alcohol containing 3% of hydrochloric acid (acid alcohol), until only a suggestion of pink remains. 4. Wash in water. 5. Counterstain with Löffler's methylene blue. 6. Wash, dry, and mount. The steaming of the slides with carbol fuchsin is most conveniently carried out by resting the slides on a piece of glass tubing bent into a V- or U-shape.

Protozoa

Leprosy bacillus differentiation.—The lepra bacillus is usually considered as being rather easily decolorized by alcohol. It is, therefore, often recommended to use 20% (or even 5%) aqueous solution of sulphuric acid for decolorization instead of the acid alcohol above recommended for tubercle bacilli. However, I have often found the leprosy bacilli as resistant to alcohol as tubercle bacilli.

Smegma bacillus differentiation.—The smegma bacillus is easily decolorized with acid alcohol and in a well decolorized smear from urinary sediment one can usually feel sure that any acid-fast bacilli are tubercle bacilli. I have had a case, however, in which numerous smegma bacilli withstood decolorization for more than 20 minutes. Nikitin's solution (Glacial acetic acid, 66; 10% acetone in alcohol, 33) decolorizes smegma bacillus in 3 minutes, but leaves the tuberculosis bacilli stained for 10 to 15 minutes.

Pappenheim's Method of Differentiation.—This is the standard method. After steaming in carbol fuchsin, pour off the stain and without washing treat film with the following: Corallin, 1 Gm.; absolute alcohol, 100 cc.; methylene blue, 0.66 Gm., and glycerin, 20 cc. The film is flooded several times with the mixture, which is allowed to drain off slowly following each application. Then wash in water and mount. The smegma bacillus is decolorized by the corallin and alcohol but the tubercle bacilli remain red.

Fontes' Method.—Fontes has devised a method of staining acid-fast bacilli which will also stain those elements which have lost their acid-fast properties. The so-called Much granules are also characteristically stained. The method is to stain the preparation with carbol fuchsin, decolorize with acid alcohol, then carry through the various steps of the Gram method, counterstaining, however, with Bismarck brown. Fontes in his method used r part of absolute alcohol and 2 parts of acetic acid as the decolorizing agent. I have obtained, however, just as satisfactory results with the ordinary acid alcohol. By this method the acid-fast tubercle bacilli show as red rods dotted with violet granules. Those which do not fully retain acid-fast properties show as zigzag violet lines.

Archibald's Stain.—This is an excellent bacterial stain and has been highly recommended by Blue and McCoy in plague work.

Solution No. 1		SOLUTION NO. 2	
Thionin	0.5 Gm.	Methylene blue	0.5 Gm.
Phenol (crystals)	2.5 Gm.	Phenol (crystals)	2.5 Gm.
Formalin	I.O CC.	Formalin	1.0 CC.
Water	22 OO I	Water	100 00

Prepare the two solutions separately and allow them to stand twenty-four hours. For use, mix in equal parts and filter. Smears, fixed by heat or otherwise, are stained for ten seconds.

Nicolle's Carbol Thionin.—To prepare this stain take 10 cc. of saturated solution of thionin in 50% alcohol and 100 cc. of 2% carbolic acid solution. This stain is good for demonstrating bacteria in tissue preparations. It is desirable to differentiate in water containing a few drops of glacial acetic acid.

Pappenheim's Methyl-Green-Pyronin Stain.—Take a very small portion of methylene green on the point of a penknife and shake it into a test tube; then take up twice as much pyronin and deposit it in the same test tube, and fill the test tube one-half full

with water. The solution should have a distinct reddish-violet color, and a drop on a piece of filter paper show a violet center and peripheral green ring. The solution should be used fresh. Stain from two to five minutes. Differentiate with a little resorcin on a penknife point dissolved in a test tube one-quarter full of alcohol. Dehydrate, clear and mount. Polymorphonuclear nuclei stain greenish; nuclei of mononuclears and plasma cells from bluish-red to dull violet; cytoplasm of lymphocytes and plasma cells purplish-red and bacteria red.

A modification of this stain is as follows: Methylgreen, 0.15; pyronin, 0.5; alcohol (95%), 5.0; glycerin, 20; and 2% aqueous solution of carbolic acid to 100.0.

Neisser's Stain for Diphtheria Bacilli.—This stain is made up in two solutions.

SOLUTION NO. 1 Methylene blue	2 cc. 5 cc. 95 cc. the alco-	SOLUTION NO. 2 Bismarck brown
ture. Filter.		

To stain: Pour on the acetic acid-methylene blue solution and allow to act from thirty to sixty seconds. Wash. Then pour on the Bismarck brown solution, and after thirty seconds wash off with water. Dry and mount. The bodies of the bacilli are brown with dark blue dots at either end.

Better preparations can be obtained by adding one part of the following solution to two parts of Solution No. 1 just before using. The formula is—crystal violet (Höchst) 1 Gm.; 95% alcohol 10 cc.; distilled water 300 cc. The following counterstain can be used in place of the Bismarck brown. Chrysoidin 1 Gm. in hot distilled water 300 cc.

Ponder's Stain for Diphtheria Bacilli.—This stain is made according to the following formula:

Toluidin blue (Grubler)	0.02 Gm.
Glacial acetic acid	ı cc.
Absolute alcohol	2 CC.
Distilled water to	TOO CC.

The film is made on a cover glass and fixed in the usual way. A small quantity of the stain is spread on the film and the cover glass is turned over and mounted as a hanging-drop preparation. Diphtheria bacilli are recognized by metachromatic granules stained with striking intensity, and diphtheroids, by their more intense staining, are sharply differentiated from ordinary cocci and bacilli which show in the preparation only as faint light-blue bodies. It is an excellent stain for bringing out the ascospores of yeasts.

Laybourn's Modification of Albert's Stain for Diphtheria Bacilli.—By substitution of malachite-green for methyl-green of Albert's formula the staining of both granules and body of cell is deepened without destroying the marked contrast between these elements. Albert's formula as modified by Laybourn:

SOLUTION No. 1			Solution No. 2	
Toluidin blue	0.15	Gm.	Iodin crystals	₂ Gm.
Malachite-green	0.30	Gm.	Potassium iodide	з Gm.
Glacial acetic acid	1	cc.	Distilled water	300 сс.
Alcohol, 95%	2	cc.		
Distilled water	100	cc.		

Let stand 24 hours and filter.

Technique.—Fix the smears by heat. Apply solution 1 for from 3 to 5 minutes. Wash with water. Then apply solution 2 for one minute. Wash, blot, and dry.

The granules of diphtheria bacilli stain black, the bars dark green, and the intermediate portions a light green. The contrast is marked. The stain is serviceable in detecting diphtheria bacilli when there are very few.

Capsule Staining.—Capsules are best observed in the hanging drop, with the greater part of the light shut off by the diaphragm.

Gram's Method.—In material in which capsules are well developed, as in pneumonic sputum, the Gram method of staining brings out the capsule perfectly.

The India-ink method of staining also shows them well.

Welch's Glacial Acetic Acid Method.—(1) Cover the preparation with glacial acetic acid for a few seconds; (2) drain off and replace with aniline gentian-violet solution; this is to be repeatedly added until all the acid is replaced; (3) wash in 1 or 2% solution of sodium chloride and mount in the same. Do not use water at any stage. The capsule stains a pale violet.

Huntoon's Method.—A 3% solution of nutrose (sodium casinate) in distilled water is cooked for one hour in an Arnold sterilizer and tubed unfiltered after adding 0.5% phenol. The organisms to be stained are mixed with a drop of this solution of nutrose, spread in thin film on a glass slide and dried in air, not fixed. The stain is as follows: phenol (2% aqueous solution), 100 cc.; acetic acid; (1% aqueous solution), 1 cc.; lactic acid (concentrated), 0.5 cc.; carbol fuchsin, 1 cc.; basic fuchsin (saturated alcoholic solution), 1 cc. The film is stained for thirty seconds, washed in water and dried. This method is not suitable for tissue smears.

Hiss' Capsule Stain.—Preparations are best made by direct films from pneumococcus exudates. Dry in air and fix by heat. Stain for a few seconds with saturated alcoholic solution of fuchsin or gentian violet, 5 cc., in distilled water, 95 cc. Flood the slide with the dye and hold the preparation for a second over a free flame until it steams. Wash off the dye with 20% aqueous copper sulphate solution. Blot (do not wash in water). By this method the capsule appears as a faint blue halo around a dark purple cell body. Better results may frequently be obtained by omitting heat fixation and by washing off the dye with the copper sulphate solution as soon as it begins to steam. Water should not be applied at any stage of the procedure.

Wadsworth's Method.—In this technique the capsule staining depends upon fixation with a good quality formalin for about 3 minutes. After fixation wash in water for 3 or 4 seconds only. Then stain as usual by any simple aniline dye or by Gram.

Flagella Staining.—Inoculate a tube of sterile water (gently) in the upper part, with just enough of an eighteen to twenty-four-hour-old agar culture to produce faint turbidity. Incubate for two hours at 37°C. From the upper part of the culture take a loopful and deposit it on a cover glass. Dry in thermostat for one to five hours or over night. All manipulations should be performed gently to avoid breaking off

. In all methods for staining flagella it is essential that the slides be scrupulously clean. Just before using flame them to remove the last vestiges of grease. The following two methods are the ones most commonly used.

Plimmer and Paine Method has proved satisfactory even in the hands of beginners. The mordant is made as follows:

Tannic acid	10	Gm.
Aluminum chloride (hydrated)	18	Gm.
Zinc chloride	10	Gm.
Rosaniline hydrochloride	1.5	Gm.
Alcohol 60%	40	cc.

The solids are placed together in a mortar, and at once, (i.e., before deliquescence) triturated with the alcohol. 10 cc. of alcohol are used first and the mass is mixed thoroughly, care being taken to smash up the whole of the zinc chloride—at this stage a homogeneous paste of a golden brown colour is obtained—the rest of the alcohol is then stirred in slowly, when the mass goes gradually into a viscous solution of deep red colour. In this state the mordant appears to remain stable for several years. For use it is diluted with 4 parts of water and allowed to stand for sixty seconds. A partial precipitation occurs. The solution is then filtered directly onto the slide and allowed to remain for a minute or two before washing. Further precipitation takes place giving the solution a metallic sheen. This precipitate, which is held in the solution as a balanced colloid, becomes adsorbed upon the flagella and periphery of the organism. No further treatment is necessary, but it is advisable to stain the body of the organism with fuchsin or methylene blue.

Preparation of the Smear.—A drop of the suspension is placed at one end of a warm slide with a 3 mm. loop. The slide is at once tilted so that the drop runs down the slide. It seems to be very important that the film should dry quickly, hence the use of a warm slide. As an alternative method, the drop may be drawn across the slide by means of a strip of paper.

**Zettnow's Method.**—Dissolve 10 Gm. tannin in 200 cc. water, warm to 50° or 60° C., and add 30 cc. of a 5% aqueous solution of tartar emetic. The turbidity of the mordant should clear up entirely on heating. The mordant keeps for months if a small crystal of thymol is added to it.

Next dissolve I gram silver sulphate in 250 cc. distilled water. Of this solution take 50 cc. and add to it drop by drop ethylamine (this comes in a 33% solution) until the yellowish-brown precipitate which forms at first is entirely dissolved and the fluid is clear. Only a few drops are required. The bacterial preparations prepared as described above are floated in a little mordant contained in a Petri dish and heated over a water bath for five to seven minutes. Take the dish containing the preparation off the water bath and, as soon as the mordant becomes slightly opalescent as the result of cooling, remove the cover-glass preparation and wash thoroughly in water. Then heat a few drops of the ethylamine silver solution upon the mordanted cover preparation until it just steams and the margin appears black. Next wash thoroughly in water and mount. This gives the most satisfactory results of any method with which I have ever experimented.

Spore Staining.—The simplest method of demonstrating spores is to stain with dilute carbol fuchsin or Löffler's methylene blue. They appear as unstained, highly refractile pieces of glass in a colored frame.

The Acid-fast Method, as used for tubercle bacilli, gives good results. The decolorizing must, however, be lightly done; otherwise the spore will lose its red stain.

Möller's Method.—Fix films by heat and then treat with chloroform for one or two minutes. Wash thoroughly and treat with a 5% solution chromic acid for one minute. Wash and then stain as for acid-fast organisms with carbol fuchsin, using a 1% sulphuric acid solution instead of the 3% acid alcohol.

Abbott's Method.—This method gives a beautiful picture. Cover the film with Löffler's alkaline methylene blue, heating the preparation to boiling from time to time—not continuously. Keep this up one minute. Wash in water and then decolorize in 2% nitric acid alcohol until the blue disappears. Wash and apply for a few seconds an eosin solution (sat. alc. sol. eosin 10 cc., water 90 cc.). Wash, dry and mount. The spores are stained blue.

Agar Jelly Staining Method of H. C. Ross.—Very clear 1.5% solution of agar is colored with Unna's polychrome methylene blue, Giemsa's solution, thionin or Gram's solution of iodine. Very thin smears of blood, faeces or gastric content sediment are made and either fixed lightly in the flame or air-dried. A drop of the melted, colored agar solution is placed on the smeared cover glass and this is mounted immediately on a clean slide. The preparation is ready for examination in about two minutes.

Polychrome (Romanowsky) Stains.—These stains are described in the section on haematology. They are also used to demonstrate malarial parasites, trypanosomes and other protozoa, spirochaetes, rickettsiae, and the intracellular inclusion bodies of certain filtrable virus infections.

Fontana's Method (Tribondeau's Modification) (for treponemata).—Methods of obtaining material for examination for T. pallidum are described in that section. The slides should be scrupulously clean, and the smears thin. Cover the dried smear repeatedly during about a minute with Ruge's solution:

Pure acetic acid	I CC.
Formalin, (40%)	2 CC.
Distilled water	TOO CC

To complete fixation alcohol is dropped on the slide and then flamed. The following mordant is then applied:

Tannic acid	5 Gm.
Distilled water	700 CC

Warm gently until steam rises and then allow to act for 30 seconds longer. Wash slide with distilled water for a few seconds and then cover slide with Fontana's solution. (To 5% silver nitrate solution ammonia is added, drop by drop, with a capillary pipette, until a sepia precipitate forms and redissolves. To this solution more silver nitrate is added until a solution is produced which remains slightly cloudy on shaking.) Cover slide with this solution and warm gently until steam arises; allow the solution to act for 30 seconds longer. Wash and dry in the air. Treponemata appear dark brown to black but fade in a few days under cedar oil or Canada balsam. The use of carbol fuchsin has been recommended in place of Fontana's silver solution.

Benian's Method (Udasco's Modification).—Mix on a cover-glass one or two loopfuls of 2% aqueous Congo red with a small amount of the serum or exudate from the lesion. Spread this mixture evenly and thinly. Dry in air. Fume over concentrated

HCl; until the film is a greenish-blue. Do not wash at any time. The treponemata appear white and unstained on a homogeneous blue ground. This method may be used on dried preparations, first treating the film with the Congo red. Alternatively the material can be mixed with an equal volume of India ink or nigrosin solution.

**Tilden's Method** (for treponemata).—This method is recommended by Noguchi The following solution is used as a fixative:

Tissue scrapings are suspended in a small amount of this solution and the mixture allowed to stand for at least five minutes. The longer the fixation the better the results; the organisms remain well preserved for at least two or three weeks. Prepare thin films from this mixture on clean slides and dry in air. If the amount of material available is very small, a drop of the fixative may be put on a slide and a drop of exudate added. Let stand for five minutes, protected from evaporation, and then spread out in a very thin film and dry in air. The film surface is flooded with a saturated alcoholic solution of gentian violet, or basic fuchsin. The slide is almost immediately washed in running water and air-dried.

Castaneda Stain (modified as described by Zinsser and Bayne-Jones) for demonstrating Rickettsiae and inclusion bodies.

## Stain (a) Buffer Solution

Potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>) r Gm. in 100 cc. distilled water Sodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>, 12H<sub>2</sub>O) 25 Gm. in 900 cc. distilled water (mixing these gives a pH of 7.5)

Add 1 cc. of formalin as a preservative

To 20 cc. of Buffer Solution (a), add 1 cc. of formalin and 0.15 cc. of (b).

The smear is stained for 3 minutes. The stain is then poured off, without washing, and the preparation is counterstained with safranin, which is allowed to remain on the slide from I to 4 seconds (never more than 5 seconds) in order to differentiate the preparation. The smear is then washed with running water and dried on filter paper.

The Rickettsiae and the inclusions may also be stained with Giemsa. The slow method described in the section on haematology is used. Special stains for the demonstration of Negri bodies are given in the section on rabies.

## II. For Protozoa

For methods of staining blood protozoa, see chapter XIII.

Unless dealing with albuminous material it is well to add a little blood serum, albumin fixative or white of egg to the preparation—about one loopful to a smear. A stock of serum or white of egg is best preserved by the addition of 2% chloroform and kept tightly corked.

Fixation of Amoebae.—Carnoy's fixative, which consists of absolute alcohol 6 parts, chloroform 3 parts, glacial acetic acid 1 part is excellent for any staining methods. It is especially useful when followed by haematoxylin. Used in the cold, it insures quick killing, rapid fixation, and fidelity of tissue elements when followed by haematoxylin stains. Immerse moist smear in fixative for 10 to 12 minutes, wash in absolute alcohol (no water) 10 to 15 minutes, in 95% alcohol 10 to 20 minutes, and then in distilled water 10 to 20 minutes. Stain.

Fixation of Intestinal Flagellates.—Nöller's method is to fix in warm concentrated bichloride of mercury solution 10 to 30 minutes, wash in gently running water for 10 minutes, drain but do not allow to dry, wash with physiologic salt solution and immerse in clear sterile serum (e.g. horse) for 5 to 15 minutes. Remove and allow to dry, wiping off back of slide first. Fix in absolute alcohol for 10 to 20 minutes, allow to dry and stain as an ordinary blood film.

Giemsa's Method.—Fix moist smears by immersion for 1 to 12 hours in a fixative made by adding 1 part of 95% alcohol to 2 parts of saturated aqueous solution of bichloride of mercury. Wash for a few seconds in water and then for about five minutes with a dilute Lugol's solution (KI, 2 Gm.; Lugol's solution, 3 cc.; water, 100 cc.). Wash in water and then in a 0.5% solution of sodium thiosulphate to remove the iodine which was used to remove the mercury. Wash in water five minutes, then stain with Giemsa's stain as used in blood work for one to ten hours. Wash and mount.

Vital Staining with Neutral Red Solution.—As a stock solution one uses a 0.5% aqueous solution of neutral red. Protozoa take a rose-pink color with a distinct differentiation between endoplasm and ectoplasm, but should the faeces be quite alkaline the neutral red will be decomposed with the formation of bilirubin-like crystals.

Panoptic Method.—The panoptic method can be highly recommended for the staining of protozoa, whether in smears or in sections.

- 1. Wright's or Leishman's stain for one minute.
- 2. Dilute with water and allow dilute stain to act for three to ten minutes. Wash in water.
- 3. Pour on dilute Giemsa's stain. Allow to stain from thirty minutes to twenty-four hours. Differentiate with 1:1000 acetic acid solution until blue stain just shows commencing diffusion into the acetic acid. Then wash in water, 95% alcohol, and absolute alcohol; treat with xylol and mount in liquid petrolatum.

With preparations other than blood smears, as sections, it is better to go from 05% alcohol to oil of origanum, then mount.

Owing to the great value of a sharp nuclear picture in differentiating amoebae it is of great importance to use some iron haematoxylin method such as that of Heidenhain described below.

Mallory's Differential Stain for Amoebae.—Stain in saturated aqueous solution of thionin for from three to five minutes. Then wash in water, clear and mount. Nuclei of amoebae are stained a brownish-red.

Iodine-Eosin Stain for Amoebae.—Kofoid's modification of Donaldson's stain has given us very satisfactory results. Mix equal parts of (1) saturated solution of eosin in normal saline (2) 5% solution of potassium iodide in normal saline saturated with iodine. This mixture when used should not be more than twelve hours old.

For examination emulsify a particle of the faeces in a small drop of saline and alongside of this a similar emulsion in the iodine-eosin stain. Cover the two drops with one cover glass. In the stained area the bacteria, faecal particles and the intestinal

yeasts (except the larger forms) stain at once. Against the pink background the protozoan cysts stand out clearly as bright spherules which soon become tinged with the iodine to varying tones of yellow, while their glycogen-filled vacuoles, when present, turn light or dark brown according to their mass. The nuclei become more clearly defined as the iodine penetrates, especially in E. coli and E. histolytica; they are detected with difficulty in this stain in E. nana.

Heidenhain's Iron-Haematoxylin Stain (as used in the U. S. Naval Medical School). (1) Prepare a thin, very homogeneous emulsion of faeces in physiological salt solution. To demonstrate trophozoites the specimen must be perfectly fresh. If no mucus is present, add a little egg albumin or use albumin fixative on the slide. (2) Spread thin films on clean glass slides (at least six), using a tooth pick or camels'-hair brush. (3) Immediately, while still moist, immerse in Schaudinn's fixing solution for one hour (or 15 minutes if kept at 50°C.). (4) Immerse for 5 minutes each in 50% and 70% alcohol, and (5) in 70% alcohol which is colored yellow-brown with iodine for 10 minutes or until film is stained brown. (6) Immerse in 70% alcohol for 10 minutes or until film is decolorized. (7) Transfer to 95% alcohol for 5 minutes to harden film. (8) Keep for 5 minutes each in 70%, 50%, and 30% alcohol and in distilled water. (9) Put in a 4% aqueous solution of iron alum (mordant) for 6 to 12 hours (cysts require the longer period). (10) Rinse in three changes of distilled water. (11) Stain 12 to 24 hours in the 0.5% haematoxylin solution (until the film becomes intensely black). (12) Rinsc in distilled water. (13) Differentiate in a freshly prepared 1% solution of iron alum until, in portions of the film of average thickness, the fine details of nuclear structure are clearly visible, but the chromatin is still jet-black. The time required is variable, and the decolorizing process must be carefully controlled. Occasionally remove the slides from the iron-alum solution, wash in water, cover with cover slips, wipe the under surface of the slide dry, and inspect under the microscope, using the <sup>1</sup>6 inch objective. If still too dense, remove cover slips by dipping quickly in water, and put back in the alum solution. The time required is usually from 20 to 45 minutes for cysts, less for trophozoites. Differentiation can be hastened or retarded by increasing (to 3% or 4%) or decreasing (to 0.5% or 0.25%) the concentration of the iron-alum solution. (14) If differentiation is adequate, put immediately in tap water to which a few drops of the staining solution has been added. (15) Wash in running tap water for 20 to 30 minutes. (16) If desired, counterstain 5 to 10 seconds in 0.5% aqueous cosin solution. (17) Put for 5 minutes each in 50%, 70%, 95%, and absolute alcohol and two changes of xylol. (18) Mount in rather thin Canada balsam.

The film must not be allowed to dry at any time during the entire process.

Solutions.—(1) Schaudinn's fixative: 2 volumes of saturated aqueous mercuric chloride solution and one volume of 95% alcohol. Just before use add 5% of glacial acetic acid.

- (2) Mordant.—4% solution of violet crystals of ferric ammonium sulphate.
- (3) Decolorizing solution.—A freshly prepared  $\tau\%$  solution of ferric ammonium sulphate.
- (4) Staining solution.—Dissolve 0.5 Gm. of certified haematoxylin in 10 cc. of alcohol by heating, and pour into 190 cc. of distilled water. The stain must "ripen" (by oxidation), by standing for several weeks before use. The ripening can be hastened by letting it stand, stoppered with cotton, in sun light in a warm place; by sucking air through the solution for 60 hours; or by adding 1 cc. of a 1 to 5000 dilution of potassium permanganate to 100 cc. of stain.

For fixing portions of a stool in bulk, Bouin's fluid is recommended, as material can be left in this fluid indefinitely without over-fixation. It is the best fixative for *Dienta-moeba fragilis*.

Bouin's fluid: Saturated aqueous solution of picric acid, 75 parts; formol, C.P., 25 parts; glacial acetic acid, 5 parts.

Johnson's Rapid Technique for Iron Haematoxylin Staining.—This was designed to save time and to avoid the necessity of controlling the decolorization by direct observation of the film during the process. Prepare thin films, spreading the material in a drop of salt solution if necessary. (1) Fix in hot Schaudinn's solution with 5% to 10% acetic acid for 10 minutes. (For Dientamocha and flagellates 15 to 20% acid gives better results.) (2) Put in 95% alcohol tinged with iodine to a port wine color for 5 minutes. (3) 70% alcohol 5 minutes. (4) Rinse in tap water 1 to 3 minutes. (4) Put in 4% iron-alum solution (mordant) for 15 minutes. (6) Rinse in tap water 1 to 2 minutes. (7) Stain with 0.5% aqueous haematoxylin for 10 minutes. (8) Decolorize in 0.25% iron-alum solution for 12 minutes. (9) Wash in running water 5 to (preferably) 30 minutes. (10) Dehydrate, clear, and mount. If differentiation is not satisfactory, it should be controlled by inspection under the microscope, as in the preceding method.

To demonstrate flagellates decolorize 2 to 4 minutes only.

Stain.—Dissolve 0.5 Gm. certified haematoxylin crystals in 10 cc. of absolute alcohol and dilute to 100 cc. with distilled water. Best results are obtained with stains which have ripened for at least 3 weeks.

*Iron-alum* solution is prepared from purple crystals. The decolorizing solution is made just before use from the mordant solution. This should be renewed whenever it shows cloudiness or a precipitate.

The method is recommended as a practical diagnostic procedure, and not as a substitute for the preceding method for accurate cytological studies. It is also applicable to tissue sections.

# D. PREPARATION OF TISSUES FOR EXAMINATION IN MICROSCOPIC SECTIONS

The most important step in the preparation of sections of tissues for histological examination is proper and immediate fixation. This step in the technique is often in the hands of the surgeon at the time of the operation or the physician at autopsy, and it should be understood by them that a satisfactory diagnosis can be made only when the pieces of tissue are at once dropped into a fixative. Various protozoa, as amoebae, disintegrate in one or two hours unless properly fixed, and body cells show degeneration after the tissues have been left without fixation for a few hours, which changes may be interpreted as pathological.

Drop into the solution slices of tissue, not more than  $\frac{1}{4}$  inch thick, as soon as cut. Leave in the fixative for twenty-four hours or longer when the specimen is to be sent away to a laboratory for diagnosis. The pathologist will attend to the other steps.

We use two fixation solutions in routine work, one of 10% formalin and one of Zenker's solution. This latter requires prolonged washing of tissues following fixation and has little advantage over formalin for ordinary purposes.

#### PREPARATION OF TISSUE

Fixation.—The piece of tissue to be fixed must not be too large. Using a sharp scalpel, or preferably a razor, a section of tissue about one-half inch square and not more

than one-fifth of an inch thick should be dropped into the bottle containing the fixative—The bottom of this bottle should have a thin layer of cotton with a piece of filter paper covering it. There should be at least 20 times as great a volume of fixing fluid as of tissue to be fixed. Delicate tissues, as pieces of gut, should be attached to pieces of glass, wood, cardboard, or blotting paper before being placed in the fixative. In fixing certain specimens of tissue, especially pieces of slit intestine, it is a good plan to lay the specimen, while wet, peritoneal side downwards, on a piece of thick dry filter paper or lintless blotting paper; this prevents the specimen from being curled up. The whole is put into the fixing fluid, and the paper removed after fixation. The number or name of the specimen may also be written on the paper.

- (1) Formalin.—The most convenient fixative for routine purposes is a 10% solution of ordinary commercial formalin (4% of formic aldehyde gas), either in water or, preferably, in 0.85% salt solution. Fixation is complete in from twelve to twenty-four hours. By placing in the incubator at 37°C., two to twelve hours in the formalin solution suffices. If fixed in the paraflin oven (56°C.), fixation is accomplished in about one-half hour. Formalin once used for fixation must be thrown away.
- (2) Zenker's fluid probably gives the best histological pictures and is the most satisfactory fixative for haematoxylin staining. It is a modification of Müller's fluid. The formula is:

Potassium bichromate	2.5	Gm.
Mercuric chloride	5	Gm.
Water	100	cc.
Just before use add glacial acetic acid 5 cc.		

Zenker's fluid fixes in about twenty-four hours. After all corrosive sublimate fixatives we should wash the tissues in running water for twelve to twenty-four hours. The precipitate of mercury in the tissues is best removed by treating the section on the slide with Lugol's solution, rather than the tissue in bulk with iodine alcohol. A saturated corrosive sublimate solution in salt solution with the additional 5% of glacial acetic acid may be used as a substitute for Zenker's fluid.

(3) Alcohol.—Where the tissue is to be examined chiefly for bacteria absolute alcohol is the best fixative. The piece of tissue should be small, not over ½ inch thick, and is to be suspended by a string to the cork so as not to lie on the bottom where the alcoholic strength tends to become weaker. Better histological details are secured by fixing for two hours with 80% alcohol and then transferring to absolute for twelve to twenty-four hours.

Note.—Most laboratories prefer to receive tissue that has been fixed in formalin. Alcohol interferes with frozen sectioning and postal regulations forbid alcohol in mail.

Dehydration.—After washing for twelve to twenty-four hours in running water following corrosive sublimate fixation, or simply washing for a few minutes after formalin, the tissues should be placed in 70% alcohol in which they may be kept indefinitely.

## E. MOUNTING AND PRESERVATION OF ANIMAL PARASITES

To Mount Small Round Worms.—Wash the hookworm, whip-worm, or filaria in salt solution, then drop in 70% alcohol containing 5% of glycerin, the glycerin-alcohol mixture being at a temperature of 60°C. When cool, pour into Petri dishes and allow the alcohol to evaporate in the 37°C. incubator.

Mount in glycerin jelly, preferably in a concave slide, and ring the preparation with gold size. The following is the formula for Kaiser's glycerin jelly: Soak 1 part of gelatin in 6 parts of distilled water for two hours. Then add seven parts of glycerin. To the mixture add 1% of carbolic acid, warm for fifteen minutes, with constant stirring, and then filter through cotton.

Nematodes.—A quick method of preparing small nematodes for examination is to fix them, after washing them in salt solution, by dropping them into hot (60° to 70°C.) 3% to 5% formalin, which keeps the worms relaxed and extended. To make a permanent mount transfer to the following solution:

Glucose syrup (glucose, 48; water, 52)	100 CC.
Methyl alcohol	20 CC.
Glycerin	10 cc.
Camphor, q.s. (a small lump for preservation).	

They may be mounted directly in this and the cover slip ringed with about 60°C. paraffin, followed with gold size. Preparations so cleared and mounted in glycerin jelly should be also ringed with paraffin or some cement.

If permanent mounts are not desired, transfer to 70% alcohol for preservation. To prepare for examination, transfer to liquid carbolic acid, which makes the worm transparent. Later return to the alcohol.

Looss has a method of first washing a small nematode or delicate fluke in salt solution. He then pours this first salt solution out of the test tube in which the washing was carried out, adds fresh salt solution, and then an equal amount of saturated aqueous solution of bichloride of mercury. The shaking is easily carried on in the test tube. After washing in water the worm is passed through alcohols, one strength of which should contain iodine. Clear in xylol and mount in balsam.

The following method gives very satisfactory results with hookworms:

- Specimens preserved in 70% alcohol:
- (a) Place directly in watch glass of carbol-xylol.
- (b) Observe on stage of dissecting microscope until completely cleared (twelve to twenty-four hours).
  - (c) Mount in chloroform balsam (saturated solution).
  - 2. Specimens preserved in 10% formalin:
  - (a) Dehydrate in absolute alcohol five to fifteen minutes.

Note.—Care should be taken not to dehydrate the specimens completely else they will appear shrivelled and distorted.

- (b) Place directly in watch glass of carbol-xylol.
- (c) Observe on stage of dissecting microscope until completely cleared (twelve to twenty-four hours).
  - (d) Mount in chloroform balsam (saturated solution).

An excellent method is that of Langeron: After washing in salt solution fix for a few hours in 5% formalin. Then transfer to lactophenol which has been diluted with an equal amount of water. Allow to remain in this solution for several hours and then transfer to pure lactophenol in which fluid the specimens are to be mounted. King with paraffin or with gold size. (To make lactophenol take 2 parts of glycerin and I part each of distilled water, crystallized carbolic acid and lactic acid.)

Flukes, cestodes, and nematodes are best stained with carmine. The following is a good formula: Dissolve, by boiling, 4 grams carmine in 30 drops HCl and 15 cc. water. Then add 95 cc. of 85% alcohol and filter while hot. Neutralize with ammonia until precipitate begins to form. Then filter cold.

1. Stain parasites taken from 70% alcohol for five to twenty minutes. 2. Differentiate in 3% hydrochloric acid. 3. Pass through alcohols to xylol and mount in balsam.

To Prepare Tape-worms.—To preserve the entire worm wash thoroughly in salt solution or running water, and fix in 10 volumes of 3% to 5% formalin. After 3 days replace with fresh formalin. To prepare a small portion for mounting, wrap a portion around a glass slide and fix in salt solution containing 2% to 5% formalin. Keep the preparation permanently in 70% alcohol. The specimen may be run through alcohols and xylol and mounted in balsam.

Larvae.—Mosquito larvae may either be prepared as are small round worms or they may be dropped into 70% alcohol at 60°C, and then passed through alcohols and cleared in xylol and mounted in balsam. Flukes and insects may require treatment with hot (60° to 70°C.) solution of 10 to 20% sodium hydrate solution. Then wash thoroughly in water and subsequently pass through alcohols to xylol and mount in balsam. Clove oil or cedar oil clears more slowly, but makes specimens less brittle than does xylol. Another satisfactory method is to drop insects or larvae into acetone at 60°C, and after being in this from one to twelve hours to clear in xylol or clove oil and mount in balsam.

Mites, Fleas and Various Small Insects.—By simply taking 1 or 2 drops of liquid petrolatum and mounting the specimen in it, then covering with a cover glass, one is able to study the details of these objects almost as well as if they were passed through acetone and xylol into balsam. Liquid petrolatum is also excellent for mounting the aërial hyphae of fungi with their sporangia as well as for Romanowsky-stained blood smears.

To Prepare Flies or Mosquitoes for Transmission through the Mails.—Wrap the insect carefully in a piece of tissue paper (toilet paper answers). Moisten sawdust with 5% carbolic acid solution and fill around the tissue paper in the box containing them.

It is very satisfactory to take a tube-form vial with a cork from the inner surface of which two small shallow holes have been bored, one containing paraformaldehyd, the other camphor. The insect is mounted upon a pin stuck in the cork, which latter is inserted and paraflined externally.

Preservation of Stools to Be Examined for Ova.—During the war, thousands of specimens of stools from all parts of the world were sent to the Naval Medical School for examination for evidence of hookworm and other intestinal parasitic diseases. The following method proved very satisfactory, the ova being well preserved for months.

- 1. Collecting: The method of collection used by Siler and Cole, U. S. Army, is recommended. Sheets of paper 14 by 14 inches are placed over each of a dozen cheap wash basins on the latrine floors. Specimens are taken from faeces deposited on the paper, and placed in a bottle. The paper and the tongue depressor used in sampling can then be thrown into the latrine and a fresh piece of paper placed over basin.
- 2. Preservation: It is recommended that each specimen bottle be half filled with 10% solution of formalin (4% solution formaldehyde). From three portions of the stool, sufficient faeces should be taken with the tongue depressors to fill the bottle

almost full. Emulsify the faeces in the formaldehyde solution at once. Replace the cork securely and mark plainly on the adhesive strip holding the cork in place, the name and rate of the patient.

# F. EQUIVALENT-NORMAL SOLUTIONS

An equivalent-normal solution contains the hydrogen equivalent of a substance, expressed in grams, dissolved in sufficient distilled water to make 1 liter. The hydrogen equivalent is the number of grams that will unite with 1 gram of hydrogen or its equivalent. For an acid, the hydrogen equivalent would be the molecular weight divided by the number of replaceable hydrogen atoms that it contains. For a base, it would be the molecular weight divided by the number of hydroxyl (OH) groups.

To make a normal (indicated by N) solution, dissolve in distilled water the proper amount of the substance, and make up the volume to exactly 1000 cc. Thus NaOII has one hydrogen equivalent: Na = 23, O = 16, and H = 1, so one dissolves 40 Gm. NaOII in distilled water, and makes the volume up to exactly 1 liter. Again, oxalic acid has the formula  $(COOH)_{2.2}H_2O$ , with a molecular weight of 126; containing two carboxyl groups, it has two hydrogen equivalents and it is necessary to divide by 2. So we dissolve 63 Gm. in water, and make up to 1 liter.

Preparation.—If a chemical laboratory is not accessible, one may prepare such solutions with an error so slight as to be unimportant in clinical work in the following manner: Select perfect crystals of oxalic acid, such as can be obtained in a drug store, and weigh out, on the most accurate apothecary scales available, 6.3 Gm. of the most perfect crystals in the bottle. Put these preferably in a volumetric flask, and make up with distilled water to r liter. The use of a measuring cylinder is less accurate. With care, this method should give N/10 oxalic acid in which the error is less than 1%.

NaOH being very hygroscopic, it is impossible accurately to prepare a normal solution directly by weighing the solid. Having N/10 acid at hand, an N/10 NaOH solution may be prepared by weighing an excess of the substance, about 5 Gm. stick caustic soda, and dissolving it in about 1100 cc. distilled water. By means of a pipette, place 10 cc. in a beaker, and add 6 drops phenolphthalein solution (1% in 95% alcohol). Fill a burette with N/10 oxalic acid, and run it into the NaOH solution until the violetpink color is just discharged. It is well to repeat the titration, and use an average. Reading the number of cubic centimeters of N/10 acid used, we can calculate the strength of the NaOH solution. If 10.5 cc. of oxalic acid solution were required, it would show that the NaOH solution was stronger than N/10, as only 10 cc. would have been necessary if it had been of N/10 strength. It is, therefore, necessary to dilute in the proportions of 10:10.5. To do this, take exactly 1000 cc. of the too concentrated NaOH solution, add 50 cc. distilled water to it, mix thoroughly, and there is then 1050 cc. of N/10 NaOH. Calculation: 10:10.5 = 1000:x.

It is sometimes desirable to use carbonate-free NaOII, and this may easily be prepared by dissolving 100 Gm. C. P. NaOH in 100 cc. distilled water. The NaOH dissolves completely, but any  $Na_2CO_3$  present is insoluble. Let it settle, or it may be centrifuged. Of the clear supernatant solution obtained after sedimentation, 55 cc. dissolved in  $CO_2$ -free water sufficient to make 1 liter will give approximately N/1 NaOH. Standardize in the usual way. Such a solution must be protected from the  $CO_2$  of the air by storing in a paraffin-lined bottle, the 2-holed rubber stopper of which bears guard tubes, and a glass tube for siphonage, the latter being lined and coated with paraffin. The guard tubes comprise one containing sodalime (or NaOH

solution) and one with H<sub>2</sub>O, and are arranged in series so that, when siphonage is established, the sodalime removes CO<sub>2</sub> from the incoming air, and then the H<sub>2</sub>O prevents the carrying of any alkali into the bottle.

Water can be freed from  $CO_2$  by vigorous boiling for 15 to 20 minutes (not longer), or by aeration for several hours, the air sucked through having been passed through NaOH solution (or sodalime tubes) and water. If the water is exposed to the air,  $CO_2$  will again be absorbed, so, if stored, it should be protected by the method described for the NaOH solution above, although the paraffin lining of tube and bottle is not essential.

Since Acidum Hydrochloricum, U.S.P., is about two-thirds water (68.1%), in order to make N/10 HCl, which would require 3.65 Gm. absolute acid per liter, it is necessary to take about three times this amount of U.S.P. acid. Take 12 cc., and add distilled water to make 1100 cc. Place 10 cc. in a beaker, add phenolphthalein solution, and titrate. If 11 cc. of N/10 NaOH were required, it would be necessary to add 100 cc. water to 1 liter of the diluted acid. Calculation: 10:11 = 1000:x. Other acid and alkali solutions can be made in the same way as are those described.

## G. DETERMINATION OF THE HYDROGEN-ION CONCENTRATION

The significance of the hydrogen-ion concentration and the nature of buffer solutions has already been discussed in the section on Acid-Base Equilibrium (p. 682). The hydrogen-ion concentration, abbreviated as  $[H^+]$ , or  $C_{H^+}$ , is a measure of the intensity of the acidity of a solution, as contrasted with the amount of acid present as determined by titration. It is commonly expressed as a fractional part of a normal solution, which contains r Gm. of H per liter. Thus the  $[H^+]$  of a o.or N solution of a strong acid, since most of the H is dissociated, is nearly o.o. Since the  $[H^+]$  of the body fluids and secretions is low, it is convenient and customary to substitute for these small fractions the logarithm of the reciprocal of the fraction; i.e., the pH.

Since appreciable although minute amounts of H are dissociated even in strong alkaline solutions (the amount diminishing as the alkalinity increases), the reaction of such solutions may also be expressed in terms of their pH. The stronger the acid the lower the pH. Thus the pH of a tenth normal solution of a strong acid would be approximately 1.0, while that of a tenth normal solution of a strong alkali would be nearly 14.

To measure the pH directly requires electrometric determinations which are impracticable for routine purposes. Instead it is customary to estimate it colorimetrically, by adding a suitable indicator to the solution and comparing the color obtained with that yielded by the same indicator in buffered solutions of varying but known pH.

Indicators. The color change of indicators is dependent upon the [H<sup>+</sup>], or its complement, the [OH<sup>-</sup>] of the solution, and for each indicator there is a definite range of pH through which there is manifested a gradual change from the full alkaline to the full acid tint. The table on p. 876 gives this range for several indicators, those italicized being the ones especially recommended for this work by Clark and Lubbs.

Buffers.—By mixing solutions of proper buffer substances in suitable proportions, mixtures of any desired pH values may be obtained. A suitable indicator is chosen from the list, and a small amount is added to each of the mixtures and to the unknown. The result is a series of graded standard tints with one of which the tint of the unknown is matched. A rough estimate of the pH of the unknown can be obtained by system-

atically testing it with different indicators, since reference to the list will show the pH at which their full acid or alkaline color may be expected.

The table on p. 877 gives the proportions in which buffer solutions must be mixed in order to produce desired pH values. The citric acid-phosphate series was proposed by McIlvaine, but we have slightly varied the proportions in order to secure colorimetric correspondence with the buffer mixtures of Sørensen and of Clark and Lubs. The boric acid, KCl-NaOH series is that of Clark and Lubs, and the resultant mixtures in it are to be diluted to 200 cc. before use. Use Na<sub>2</sub>HPO<sub>4.2</sub>H<sub>2</sub>O (Sørensen's phosphate) in M/5 strength; M/10 citric acid; a solution containing 12.4048 Gm. boric acid and 14.912 Gm. KCl per liter; and M/5 NaOH.

T. V. 4	-II wa ma	Color	change
Indicator	pH range	Acid	Alkaline
Thymol blue	1.2- 2.8	Red	Yellow
Töpfer's reagent (dimethylamino-azobenzol)	2.9-4.0	Red	Yellow
Brom-phenol blue	3.0-4.6	Yellow	Blue
Congo red	3.0- 5.0	Blue	Red
Methyl orange	3.1-4.4	Red	Yellow
Brom-cresol green	4.0- 5.8	Yellow	Blue
Methyl red	4.4-6.0	Red	Yellow
Litmus (azolitmin)	4.5-8.3	Red	Blue
Cochineal	4.8-6.2	Yellow	Lilac
Brom-cresol purple	5.2- 6.8	Yellow	Purple
Alizarin	5.5-6.8	Yellow	Blue
Brom-thymol bluc	6.0- 7.6	Yellow	Blue
Neutral red	6.8 8.o	Red	Orange
Phenol red (phenol-sulphonephthalein)	6.8-8.4	Yellow	Red
Cresol red	7.2-8.8	Yellow	$\operatorname{Red}$
Thymol blue	8.0-9.6	Yellow	Blue
Cresol phthalein	8.2- 9.8	Colorless	$\operatorname{Red}$
Phenolphthalein	8.3-10.0	Colorless	Red
Thymol phthalein	9.3-10.5	Colorless	Blue

The chemicals employed in the preparation of such solutions must be specially purified—a task probably not within the ability of the usual clinical laboratory. The NaOH solution must be prepared and stored as indicated on page 874. We will not give the details of this purification inasmuch as the laboratory with the equipment and experience necessary for this will have access to the literature. The buffer solutions and indicator solutions are readily purchasable, and, for the usual laboratory, we would advise that they be so obtained.

Sørensen's M/15 phosphate mixtures are also much used for pH range from 5.8 to 8.2. Dissolve 9.08 Gms. of KH<sub>2</sub>PO<sub>4</sub> in one liter of water.

Dissolve 9.47 Gms. anhydrous Na<sub>2</sub>HPO<sub>4</sub> in one liter of water.

Prepare mixtures according to the table on p. 878.

рН	$ m M/_5$ Phosphate, cc.	M/10 Citric acid, cc.	На	Boric acid- KCl, cc.	M/5 NaOH, cc.
2.2 2.4 2.0 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.0 4.8 5.0 5.2 5.4 5.8 6.0 6.8 7.0 7.2 7.4 7.0 8.0 8.0 8.0 8.0 8.0 8.0 8.0 8	0.40 1.24 2.18 3.17 4.11 4.94 5.70 6.23 6.77 7.40 7.99 8.42 8.82 9.50 9.86 10.52 10.94 11.37 11.85 12.43 12.82 13.22 14.50 15.40 10.47 17.12 17.78 18.45 18.45	19.60 18.76 17.82 16.83 15.89 15.06 14.30 13.77 13.23 12.60 12.01 11.58 11.18 10.50 10.14 0.48 9.00 8.63 8.15 7.57 7.18 6.78 5.50 4.60 3.53 2.88 2.22 1.55 0.85	7.8 8.0 8.2 8.4 8.6 8.8 9.0 9.2 9.4 9.6 9.8 10.0	50.0 50.0 50.0 50.0 50.0 50.0 50.0 50.0 50.0 50.0	2.61 3.97 5.90 8.50 12.00 16.30 21.30 26.70 32.00 36.85 40.80 43.90

**Procedure.**—Select test tubes of clear resistant glass, uniform in diameter, by introducing exactly 10 cc. of water into each, and selecting those in which it rises to the same height. It is well to make permanent marks on these tubes at 5 cc. and 10 cc. Into a series of these tubes introduce 10 cc. (or 5 cc.) of standard buffers solutions selected from the range of the pH anticipated in the solution to be tested. In 3 additional tubes put an equal volume of this solution. It should be clear, and diluted up to 5 or 10 times with distilled water if highly colored. To each standard tube and to one tube of the unknown add with a pipette or medicine dropper held vertically equal volumes of a suitable indicator solution and mix.

For the indicators that they recommend Clark and Lubbs suggest 5 drops for 10 cc. of solution. The concentration of the indicator solution should be 0.02% for cresol red,

pH 20°C.	M/15 Na <sub>2</sub> HPO <sub>4</sub> cc.	M/15 KH <sub>2</sub> PO <sub>4</sub> cc.	pH 20°C.	M/15 Na <sub>2</sub> HPO <sub>1</sub> cc.	M/15 KH <sub>2</sub> PO <sub>4</sub> cc.
5.8 5.9 6.0 6.1 6.2 6.3 6.4 6.5 6.6	8.0 9.0 12.2 15.3 18.6 22.4 26.7 31.8 37.5 43.5 49.6 55.4	92.0 91.0 87.8 84.7 81.4 77.6 73.3 68.2 62.5 56.5 50.4 44.6	7.0 7.1 7.2 7.3 7.4 7.5 7.6 7.7 7.8 7.9 8.0	61.1 66.6 72.0 76.8 80.8 84.1 87.0 89.4 91.5 93.2 94.7	38.0 33.4 28.0 23.2 19.2 15.9 13.0 10.6 8.5 6.8 5.3 4.2

phenol red, methyl red and cresol phthalein; for the others, 0.04%. The solutions are prepared by grinding 0.1 Gm. of the dry dye in a mortar with N/10 NaOH, using the following volumes of alkali: For cresol red, 2.88 cc.; phenol red, 3.1; methyl red, 4.07; brom-phenol blue, 1.64; brom-cresol purple, 2.78; and thymol blue, 2.38. These are diluted with distilled water to 500 cc. to make a 0.02% solution; to 250 cc. if a 0.04% solution is desired.

Precise determinations require many precautions as regards background, source of light, exclusion of adventitious light, etc. It is very advantageous to use a wooden comparator block, as illustrated. We prefer to use diffuse day light if possible, and to



Fig. 206.—Block comparator. (Courtesy of A. H. Thomas Co.)

back the comparator block with ground glass plate or with thin plain unglazed white paper. In colorimetric comparisons the unknown and standard solutions must be balanced as regards any factors that would affect the shades of color compared. These are particularly intrinsic color or turbidity in the unknown solution and the thickness of the water columns. To avoid these errors, arrange the tubes in the block thus: In the two lateral spaces next to the observer put the tubes containing the unknown solution without indicater: in the middle space, a tube of water. In the spaces next to the light put, in the middle space the unknown solution containing indicater: in the light put, in the middle

space the unknown solution containing indicater; in the lateral spaces standard tubes, changing the latter until the one is found which most nearly matches the color of the unknown. Interpolate if necessary. One must not rely on a color match with the

end tube of a standard series. The result must be checked by using another series so selected that the pH of the unknown falls well within its range.

It is imperative that the glass ware be chemically clean, that the chemicals used be the purest obtainable, and that the distilled water be neutral and free from CO<sub>2</sub>. If the standard solutions are sealed in ampoules of resistant glass having exactly the same diameter as the tubes used for the unknown solutions, and if they are kept in a dark cool place when not in use, they may usually be used for several months or more, but the colors should be checked occasionally.

Examination of Urine.—The pH of the urine changes very rapidly on exposure to the air, because of the escape of CO<sub>2</sub>. To get really accurate determinations it must be preserved (and if possible be collected) under oil, and examined under oil. In a tube put 8 cc. of distilled water, add the indicator solution, and cover with oil. With a pipette introduce 2 cc. of urine, keeping the tip of the pipette under the surface of the water. Then proceed as above. The usual pH is about 6, the range being from about 4.8 to 7.5. Bromcresol green or purple may be used for acid specimens, brom-phenol blue for average aciditics, and phenol red for alkaline specimens.

Standardization of Media.—Determination of the pH has practically supplanted titration as a means of adjusting the reaction of culture media. It is more precise and equally simple after the standard comparison tubes have been prepared. Bromthymol blue in 0.04% alcoholic solution is a satisfactory indicator for the range usually required. Phenol red is often used, in 0.02% solution. Since the object is to bring the medium to a desired pH rather than to determine its initial reaction, the procedure previously described is altered as follows:

Boil a portion of the medium to expel  $CO_2$  and cool. Put 10 cc. in each of three tubes. To one add 5 (or 10) drops (or 0.5 cc.) of indicator solution and place in the middle space of the comparator block. In the lateral spaces put standard tubes having the pH desired and containing the same amount of indicator. Back them with tubes containing medium without indicator, and back the center tube with a tube of water. From a burette add N/10 NaOH (or N/10 HCl if the medium is too alkaline) until the color matches that of the standard tube. Note the volume added (V).

Since V cc. of N/10 NaOH were required to bring 10 cc. of medium to the desired reaction, then V cc. of normal alkali will adjust 100 cc. of medium. The amount of normal alkali required to adjust the entire quantity of medium is calculated by dividing the volume of the latter by 100 and multiplying by V.

Since the addition of alkali to the medium often clouds it by precipitating phosphates and necessitates filtration, it may be more convenient first to add roughly a moderate excess of alkali to the entire quantity (till a distinct pink is obtained with phenolphthalein), filter, and then bring back to the desired pH with N/10 HCl, as above described. For ordinary routine purposes a pH of 7.4 is suitable. If great precision is required, the reaction should be tested again after sterilization and adjusted if necessary. Comparator outfits with standard color tubes can be purchased.

The bicolor method of determining the pH has many advantages over the use of buffer solutions. The standard solutions are more permanent, are less affected by variations of temperature, and errors due to impurities in the buffers are climinated, although pure indicator solutions are essential. The method has been elaborated by Gillespie and by Hastings et al. (1925) and applied to determinations of the pH of the urine and blood plasma, and yields accurate results. (For details consult original article or Peters and Van Slyke, Quantitative Clinical Chemistry.)

Barnett and Chapman introduced a relatively simple procedure which illustrates the principles of the method and is sufficiently accurate for most practical purposes.

Place 18 test tubes in rack in 2 rows of 9 each. Beginning at the left in the front row, place 1 drop of indicator in the first tube, 2 in the second, and increase by one drop in each succeeding tube as one passes to the right. Treat the rear row similarly, but begin at the right and pass to the left. To each tube in the front row, add 1 drop (2 to 3 drops for the thymol-blue series) N/20 NaOH; to each tube in the rear row, add 1 drop N/20 HCl (use 1 drop 2% KH<sub>2</sub>PO<sub>4</sub> for thymol-blue series instead of HCl).

Fill all tubes to 5-cc. level with water. This standard series is viewed from the front in such a manner that the line of vision traverses 2 tubes—one in the front row and its partner in the rear row—the total amount of indicator in each pair being 10 drops. The composite colors form a graded series, and the following table shows the pH values represented by each pair. The methyl red solution is prepared by grinding in acid-free alcohol until dissolved, and then diluting 3 volumes with 2 volumes of H<sub>2</sub>O. The preparation of the other indicators is given above.

		pH valı	ies with	
Drop ratio (Front: Back)	Methyl red (0.008°c)	Brom-cresol purple (0.012%)	Phenol red (0.004%)	Thymol blue (0.008%)
1:9	4.05	5.3	6.75	7.85
2:8	4 · 4	5 · 7	7. T	8.2
3:7	4.6	5.9	$7 \cdot 3$	8.4
4:6	4.8	6. т	7 · 5	8.6
5:5	5.0	6.3	$7 \cdot 7$	8.8
6:4	5.2	6.5	7 · 9	9.0
7:3	$5 \cdot 4$	6.7	8.I	9.2
8:2	5.6	6.9	8.3	9.4
9:1	5.95	7.2	8.65	9.75

In a tube containing 10 drops of indicator solution put sufficient of the unknown solution to bring the volume to 5 cc. and mix. Put in a comparator block and back with two tubes of water. Also put a pair of standard tubes in the block and back with a tube containing the unknown solution without indicator. Change the pairs of standard tubes until a pair is found which matches the color of the unknown. Since this procedure necessitates looking through three tubes in series, the openings for inspecting the tubes must be drilled lengthwise through the comparator block.

### H. COLORIMETRIC DETERMINATIONS

There are two types of colorimeters in general use; in one, the plunger type, the intensity of the colors of the two solutions is matched by varying the depths of the solutions; in the other this is accomplished by diluting one of the solutions, the depth of the fluid traversed by the line of vision remaining constant.

With the plunger type the relative concentrations of the substance will vary inversely as the readings (depths), and the calculation follows the general formula:

$$\frac{\Im}{U} \times F = X$$

in which S and U are, respectively, the readings of the standard and unknown solutions when colors are matched, F is a factor, and X is the sought result. F is constant under

the conditions of each determination, and its value is determined by three considerations, viz.: (1) The actual amount (in terms of X) of substance (nitrogen, uric acid,

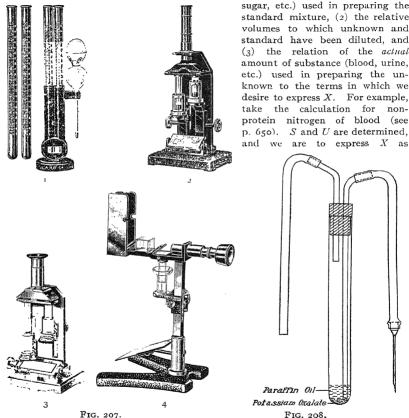


Fig. 207.—Types of colorimeter. (1) Myers; (2) Duboscq; (3) Duboscq (Pellin); (4) Bock-Benedict. (Courtesy of A. H. Thomas Co.)

Fig. 208.—Tube used in collecting blood. (Journal Biological Chemistry, 30, 289, 1917.)

milligrams per 100 cc. blood.

$$\frac{S}{U} \times 0.3 \times \frac{50}{100} \times \frac{100}{0.5} = \frac{S}{U} \times 30 = \text{milligrams non-protein nitrogen per 100 cc. blood.}$$

(1) We have used 0.3 mg nitrogen (not ammonium sulphate); (2), standard diluted to 100 cc. and unknown to 50 cc.; therefore multiply by 50/100 (or divide by 2); (3),

 $_{5}$  cc. filtrate represents 0.5 cc. whole blood, and X is to be expressed in terms of 100 cc.

With the dilution type of colorimeter, the same reasoning applies, but we have here a direct proportion, and the general formula for the calculation becomes  $U \times F = X$ , if, as is usually the case, it is the unknown solution which is diluted.

It has been advised usually to set the standard solution at a fixed point and vary the depth of the unknown to match it. The calculation may be simplified by setting the unknown at some appropriate height and varying the depth of the standard. In the example above, e.g., if the unknown is set at 30, the reading of the standard gives the value of X directly.

Permanent standards of tinted glass or of stable solutions for practically all the examinations can be purchased, but very few are satisfactory. It is usually preferable to prepare the standard at the time of the test, using the same reagents as for the unknown. Standard and unknown solutions should be handled in the same way and finished practically simultaneously. In using a colorimeter take precautions against retinal fatigue and adventitious light. Adjust the scales if necessary so that they read zero when the cups are elevated so that they just touch the plungers, or determine a correction factor. Make sure that the fields are equally illuminated and check by reading the standard against itself. If the field is divided by a line, this line must be sharply focussed and the halves of the field made equal in area.

If a regular colorimeter is not available, Nessler tubes or even graduated cylinders or test tubes can be used quite satisfactorily for approximate results provided their internal diameter is identical, the length of the column of fluid being measured by a ruler.

### I. DISINFECTANTS AND DISINFESTANTS

By disinfection is meant the destruction of injurious bacteria; by sterilization, the destruction of all living things. Germicides are substances which kill disease-producing bacteria. The term "antiseptic," which has been restricted technically to those substances which are inimical to the growth of bacteria, has come to have a wider meaning. The layman will generally buy an "antiseptic" with the idea in mind that it will prevent infection by killing germs. In line with a judicial decision that "Language used in the label is to be given the meaning ordinarily conveyed by it to those to whom it is addressed," the Bureau of Chemistry, U. S. Department of Agriculture, has held that the term "antiseptic," when used in the labeling of medicinal substances, "is objectionable unless the preparation, when used as directed, will actually render pathogenic microorganisms innocuous."

Deodorants may or may not be antiseptic or germicidal. An insecticide may or may not be a germicide and vice versa.

In disinfection we must consider:

- 1. Strength of solution. It must always be kept in mind that the strength of a germicidal solution when added to an equal amount of material to be disinfected is reduced in strength one-half. Thus 1 pint of a 5% compound cresol solution added to 1 pint of faecal material has a disinfecting effect of a 2.5% solution.
- 2. Time of application. A common mistake is to consider a few minutes as sufficient for contact of germ-containing material with the disinfectant. In the faeces-cresol mixture above noted the action of the disinfectant should continue at least one hour before emptying the vessel.

- 3. Nature of medium in which disinfectant acts. Germicidal agents are much less effective against bacteria contained in material rich in organic matter than when in pure water.
- 4. Temperature. Disinfecting solutions show greater power as the temperature rises, and act less efficiently in the cold. At 39°C., they are active.

By Coefficient of Inhibition we mean time and concentration necessary to prevent development of bacteria.

By Inferior Lethal Coefficient we mean time and concentration necessary to kill non-spore-bearing bacteria.

By Superior Lethal Coefficient we mean time and concentration necessary to kill spore-bearing bacteria.

Phenol Coefficients.—In determining the germicidal strength of a disinfectant against any given organism it is compared with that of phenol. The Bureau of Chemistry, Department of Agriculture, determines the phenol coefficients of disinfectants under the provisions of the Food and Drugs Act and the Insecticide Act. They are expressed as the B. typhosus phenol coefficient, S. aureus phenol coefficient, etc., depending upon the organism used in the determinations. If more powerful than phenol under the conditions of test the coefficient will be greater than 1. The method employed by the Bureau of Chemistry is based on the Rideal-Walker and the Hygienic Laboratory methods but differs from both in some respects.

### Disinfectants May Be (A) Physical, (B) Chemical

### (A) Of the physical disinfectants we have:

- 1. Sunlight. The red and yellow rays are practically inert, the ultra-violet most active. Direct sunlight kills non-spore-bearing pathogenic organisms in from one to several hours depending upon moisture, temperature and other conditions. Exposure equivalent to 30 hours sunlight is usually required to kill anthrax spores.
  - 2. Burning is effective when practicable.
- 3. Boiling is efficient. Non-spore-bearing bacteria are killed almost instantly by a boiling temperature, but spores may resist destruction for many hours at 100°C. One must remember that the boiling point is lower at mountainous elevations.
  - 4. Steam is extremely efficient when penetration is insured.
- (B) Chemical Disinfectants.—Bichloride of mercury is usually sold in the form of antiseptic tablets. As a disinfectant for the infectious diseases it is usually used in a strength of 1-1000. The solution should be made in a wooden, enameled or earthenware vessel. As bichloride forms inert albuminates it should not be used in disinfection of sputum, faeces or any albuminous excreta. It must be remembered that bichloride is a mordant so that any stains in soiled clothing will remain permanent. For disinfection of clothing the material should be left in 1-1000 bichloride for one hour. Dishes for food should never be disinfected in bichloride on account of the danger from poisoning. Floors and walls may be disinfected with 1-1000 bichloride applied with a mop. Allow the solution to dry on the floor or walls.

Solution of formaldehyde, U.S.P., contains not less than 37% of formaldehyde. A 5% dilution in water (50 cc. sol. formaldehyde, 950 cc. water) makes a satisfactory disinfectant for soiled clothing. It is also valuable for albuminous material. The disinfectant must act in a strength of 5% so that if  $\tau$  pint of faeces is to be disinfected we should add to it a  $\tau 0\%$  dilution of the official solution and allow it to act for one hour.

Fumigation with formaldehyde is employed only when the object is to destroy bacteria as the gas is valueless as an insecticide. Such fumigation is now seldom considered necessary in public health practice. Formaldehyde is efficient as a surface disinfectant when the temperature is above 50°F, and the air contains at least 60% of moisture. Owing to its lack of penetration the gas is not efficient for the disinfection of mattresses, or similar articles.

A convenient method of formaldehyde fumigation is to pour 500 cc. of Solution of Formaldehyde on 250 Gm. of barium dioxide or potassium permanganate for each 1000 cubic feet, allowing exposure for 6 to 12 hours.

In employing this method, take a pan partly filled with water. Place in this a second metal or glass receptacle containing the barium dioxide or potassium permanganate and pour in the Solution of Formaldehyde. The gas is generated in great amounts in a few seconds. The receptacle should be large enough to contain 10 times the volume of the Solution of Formaldehyde, as there is a tendency for the mixture to foam over the sides of the container.

Another practical method is to spray sheets with formaldehyde solution. The solution (37%) should be sprayed on sheets suspended in the room in such a manner that the solution remains in small drops on the sheet. Spray not less than 10 ounces of Solution of Formaldehyde for each 1000 cubic feet. Used in this way a sheet will hold about 5 ounces without dripping or the drops running together. The room must be sealed very tightly in disinfecting with this process and kept closed not less than twelve hours. The method is limited to rooms or apartments not exceeding 2000 cubic feet. The formalin may also be sprayed upon the walls, floors, and objects in the room.

Phenol.—It is soluble in water to the extent of about  $5^{\circ}$  and in such strength it is an efficient disinfectant. The solution should be made with hot water.

In standardizing disinfectants phenol is used as the standard. It is expensive, however, and there is often difficulty in making up satisfactory solutions. More efficient and more convenient is the *Liquor Cresolis Compositus*, *U.S.P.* This may be prepared by mixing equal parts of cresol and soft soap. This has a value according to tests made in the Hygienic Laboratory of 3, making it in tests without organic matter three times as efficient as phenol. Under similar conditions lysol had a value of 2.12, creolin 3.25 and trikresol of 2.62.

Equal parts of a 5% solution of Liq. Cresol. Co. and the faeces, urine or sputum to be disinfected is satisfactory for disinfection provided the mixture is allowed to stand for one hour. Here we would have the effect of a 2.5% solution. Liq. Cresol. Co. (5%) is an excellent disinfectant for contaminated bed clothing, etc. It is also most suitable for the disinfection of floors and walls.

Sulphate of copper.—This salt has a remarkable effect on certain species of algae so that in strengths of 1 to 1,000,000 it is destructive.

Hydrogen dioxide.—A  $2^{\circ c}$  solution will kill anthrax spores in three hours. It is useful in treatment of anaerobic infections, as with the gas bacillus. When hydrogen dioxide is used in the presence of blood or pus, the catalase of the latter rapidly decomposes the  $H_2O_2$  so that the disinfecting power rapidly disappears. The quality of hydrogen peroxide cannot be depended upon on account of its rapid deterioration.

Lime.—It must be remembered that air-slaked lime is inert as a disinfectant. For disinfecting faeces freshly prepared milk of lime is excellent. It is made by mixing unslaked lime with four times its volume of water. An equal quantity should be added to the faeces to be disinfected.

CHLORINE 885

Chlorinated lime.—This can be purchased in air-tight containers and when the package is opened it should give off a powerful odor of chlorine. Frequently samples fail to yield 30% of available chlorine (the U.S.P. requirement).

For a working disinfectant solution add 1 pound to 2 gallons of water, allow insoluble matter to settle and decant the clear liquid. This is satisfactory for mopping floors and for disinfecting faeces, sputum and urine, equal parts of the excreta and disinfecting solution being mixed and allowed to stand for one hour.

Chlorine.—The water supply for communities is generally treated with chlorine gas for the destruction of the typhoid-colon group. The available chlorine of chlorinated lime and of sodium hypochlorite is also utilized for the disinfection of drinking water. The amount of chlorine necessary to destroy pathogenic organisms will vary from 0.2 part to many parts per million depending upon the source of the water, the season, degree of contamination, the amount of organic matter present and other factors. It is considered that the presence of o.r part of residual chlorine per million parts of water fifteen minutes after chlorination is an indication that the pathogenic bacteria have been killed. This small excess of chlorine disappears shortly and has no effect on the taste of the water. For determining the amount of residual chlorine, a reagent consisting of 0.1% of orthotoluidine in a 10% solution of sulphuric acid or hydrochloric acid is employed. I cc. of this solution is added to 100 cc. of the water to be tested. The color developed in the sample is compared with a series of quantitative colorimetric standards. A non-fragile colorimetric device calibrated for 0.05 part of free chloring per million, to be used with orthotoluiding, is sold in this country for the convenience of those who have not a series of colorimetric standards available. In the field with troops, overchlorination followed by the use of sodium thiosulphate as an antichlor has been practiced in the absence of facilities for the accurate determination of the amount of residual chlorine. Where water is turbid treatment with some precipitating agent like alum is required preliminary to chlorination. Automatic chlorinators for use with either chlorine or chlorinated solutions are now available. They are especially useful for ships operating in fresh water and in the case of water supplied by lighters as the water may be disinfected automatically as it is being pumped on board. Lelean recommends the addition of 2 grams of good quality bleaching powder (chlorinated lime) to the contents of an ordinary water cart which holds 110 gallons of water. This is actually about 123 parts per million of chlorine so that allowing for possible deterioration we can count on 1 part per million being operative. Other agents used for disinfection of small quantities of water are "halazone," sodium bisulphite and calcium permanganate. Halazone tablets have been giving excellent results in the sterilization of the contents of drinking bottles. It must always be remembered that boiling the water is the method of sterilization to be employed when practicable.

Eusol.—A solution containing 0.27% hypochlorous acid and known as cusol has been highly recommended in the treatment of gas gangrene wounds. To make it, put 12.5 grams chlorinated lime (bleaching powder) in a Winchester quart flask and cover with a liter of water. After thorough shaking add 12.5 grams of boric acid. After again shaking the mixture should stand for a few hours and then be filtered through cotton wool. The clear solution is eusol. It must be kept in tightly closed bottles.

Chloramine-T.—This chlorine antiseptic is more stable than hypochlorite solutions and can be used in greater concentration. It is non-toxic and readily soluble in water. It is usually used in 2% solution in the treatment of wounds. Gauze which has been

impregnated with a 5% solution and dried can be used in light packing of wounds. In the eye, 0.1% (1-1000) in physiological saline is efficacious and non-irritating.

Dichloramine-T.—This, like chloramine-T, is a crystalline substance, but is practically insoluble in water. It is a very active germicide. In use it is dissolved in chlorinated eucalyptol or better still chlorinated paraffin wax (chlorcosane). For treatment of infected wounds it is used in 6.5 to 10% strength, the chlorinated oil solution of the antiseptic being sprayed on the wound or gauze covering the wound.

Acriflavine.—Of the dyestuffs recommended as germicides this is better adapted to the purpose than malachite green or brilliant green. Acriflavine, or flavine as it is also called, acts more efficiently in serum mixtures than in aqueous ones and is less injurious to tissue than most other antiseptics. It is generally used in 1–1000 solution in salt solution and makes a good wet dressing when gauze is soaked in such a solution.

Dakin's solution.—The best known and most widely used of the disinfectants of the chlorine group is a neutral sodium hypochlorite solution called Dakin's solution. This contains not more than 0.5% or less than 0.45% NaOCl in a neutral solution.

The usual method of preparing Dakin's solution is to make a 1.4% solution of anhydrous Na<sub>2</sub>CO<sub>3</sub> and slowly introduce chlorine gas from a cylinder provided with a flow meter, adding 4.8 Gm. or 1600 cc. of gas per liter of solution. To titrate, remove 10 cc., add 25 cc. of water, 5 cc. of 10% KI solution and 2 cc. glacial acetic acid. Add from a burette N/10 sodium thiosulphate solution until brown color is discharged. Amount should be from 12.1 to 13.4 cc. If necessary, dilute with 1.4% Na<sub>2</sub>CO<sub>3</sub> solution. Test reaction with phenolphthalein as described below.

The following method of preparing Dakin's solution was formerly used in the chemical laboratory of the Naval Medical School and is recommended if chlorine gas in cylinders is not available.

(A)	Bleaching powder	100 Gm.
	Water	1000 CC.
	Shake to mix thoroughly.	
(B)	Sodium carbonate	45 Gm.
	Sodium bicarbonate	48 Gm.
	Water	1000 cc.
	Dissolve completely.	

Mix A and B and shake vigorously for 5-ro minutes or allow to stand in a closed container a few hours. Then filter. This filtrate is the Dakin's solution which will be neutral to solid phenolphthalein (flash of red with alcoholic solution), but will contain about two to three times the amount of NaOCl required. Determine exact per cent of NaOCl and dilute to proper strength as follows:

Put 10 cc. of the filtered Dakin's solution in a 100-cc. volumetric flask. Add 20 cc. of 10% KI and 2 cc. of glacial acetic acid. Dilute to mark with  $\rm H_2O$  and mix thoroughly. Put this wine-colored solution in a burette. In Erlenmeyer flask put 5 cc. of N/10 sodium thiosulphate (24.8 Gm. Na<sub>2</sub>S<sub>2</sub>O<sub>3.5</sub>H<sub>2</sub>O per liter of H<sub>2</sub>O) and add 2 cc. of starch paste for an indicator. (The starch paste is best made by mixing 1–2 Gm. of starch with about 10 cc. cold water and pouring this into 90 cc. of boiling water.) From the burette run this solution into the 5 cc. of thiosulphate until a faint blue color results. This is the end-point. Take the reading on the burette and calculate the per cent NaOCl as follows:

### Calculation:

$$\frac{50}{\text{per cent NaOCl}}$$
 = the number of cc. of above solution required to dilute to

100 cc. to make 0.5% NaOCl.

### Example:

20 cc. of solution from the burette were required to reach the end-point.

$$\frac{18.615}{29} = 0.64\% \text{ NaOCI.}$$

$$\frac{50}{2.64} = 77.88 \text{ cc. So take } 77.88 \text{ cc. of the Dakin's solution and dilute to 100 cc.}$$

This will give 0.5% NaOCl, the required per cent.

Equations involved in the above are as follows:

$$\begin{aligned} & \text{CaCl}(\text{OCl}) + \text{Na}_2\text{CO}_3 = \text{NaOCl} + \text{NaCl} + \text{CaCO}_3 \\ & \text{NaOCl} + \text{HC}_2\text{H}_3\text{O}_2 = \text{HOCl} + \text{Na}\text{C}_2\text{H}_3\text{O}_2 \\ & 2\text{KI} + 2\text{HC}_2\text{H}_3\text{O}_2 = 2\text{HI} + 2\text{KC}_2\text{H}_3\text{O}_2 \\ & \text{HOCl} + 2\text{HI} = \text{I}_2 + \text{HICl} + \text{H}_2\text{O} \\ & \text{I}_2 + 2\text{Na}_2\text{S}_2\text{O}_3.5\text{H}_2\text{O} = \text{Na}_2\text{S}_4\text{O}_6 + 2\text{NaI} + 1\text{oH}_2\text{O} \end{aligned}$$

### Disinfestants

By disinfestants we mean those agents used for the destruction of rodents and insects.

### Gaseous Disinfestants

Among the fumigants effective against both rodents and insects we have sulphur dioxide, hydrocyanic acid gas, Zyklon-B which was authorized for use in the Public Health Service in 1926, and cyanogen chloride gas. These are destructive to all forms of animal life. Hydrocyanic acid gas and cyanogen chloride gas do not injuriously affect merchandise, textiles, etc. and require less time for exposure than sulphur dioxide. Although sulphur dioxide has the manifest disadvantages of rotting fibres of textiles and bleaching certain dyed fabrics, its safety of application makes its use preferable to cyanide fumigants by those inexperienced in large scale fumigation. Prior to fumigation of vessels the crew should be mustered and all absentees accounted for in order that none might remain in the compartments to be fumigated. Thereafter measures should be taken to prevent the entry of any unauthorized person to such compartments until the medical officer has pronounced them safe for occupancy. In fumigating for rats a check is made after fumigation to insure that no rats remain alive. If necessary, fumigation is repeated. After fumigation, hatches, ventilators, doors, etc. are opened from the outside. The Public Health Service uses "Aerothrusts" (portable gasoline motors with airplane blade fans to remove the gas from holds and spaces having poor

### DISINFECTANTS AND DISINFESTANTS

ventilation). They do not enter the superstructure for at least  $\tau_5$  minutes after opening up and do not enter the holds until they have been open an hour. In addition a tame rat in a cage is lowered to the bottom of the hold and left for at least five minutes to test for hydrocyanic acid gas or cyanogen chloride gas. If the rat is unaffected, the medical officer goes personally or observes one of his men go through all the compartments before they are declared safe for occupancy. Men doing this carry an anticyanide gas mask in the alert position, are equipped with searchlights, and are watched from the deck.

Sulphur dioxide.—For destruction of rats 5 pounds of roll or sublimed sulphur are burned per 1000 cubic feet of space. Shallow pans should be used for the sulphur which is sprinkled with alcohol and ignited. Precautions should be taken against fire by elevating the pans by means of bricks, etc., in a larger vessel containing water. Exposure for six hours is necessary. Two pounds of sulphur per 1000 cubic feet with two hours exposure are sufficient for destruction of mosquitoes, whereas for body lice four pounds per 1000 cubic feet with six hours exposure are required. Liquefied sulphur dioxide may be employed instead of burning sulphur, two pounds of the gas being substituted for each pound of sulphur. Machinery may be protected from the action of sulphur dioxide by coating the metal parts with lubricating grease. If clothing is washed immediately after sulphur fumigation the rotting effect will be lessened.

Hydrocyanic acid gas.—For destruction of rats 5 oz. sodium cyanide of high purity, 7½ oz. commercial sulphuric acid (60B) and 10 oz. water are required for each 1000 cubic feet of space. Exposure for two hours is necessary. The water is placed in a crock and the acid is run in cautiously immediately before fumigation in order to take advantage of the heat generated. The sodium cyanide, contained in a cloth bag, is dropped in the diluted acid by an operator who wears a special anti-cyanide gas mask. The ordinary military gas mask does not protect against hydrocyanic acid gas and cyanogen chloride. Sodium cyanide "eggs" of the proper weight are to be preferred to the loose cyanide. Liquid hydrocyanic acid in cylinders is efficient but dangerous to transport. For generation of HCN for destruction of insects Creel and Faget employed materials in the following proportions: potassium cyanide 1 part, commercial sulphuric acid (60B) 2 parts and water 2½ parts by weight. The following amounts of potassium cyanide per 1000 cubic feet of space were recommended: for mosquitoes 0.4 ounce, 15 minutes exposure; for bed bugs 5 ounces, exposure for one hour; for body lice 10 ounces, two hours exposure; for roaches 10 ounces, one hour exposure.

The Public Health Service now use Zyklon Discoids (a trade name of American Cyanamid & Chemical Corporation). These contain 5 per cent chlorpicrin (a lacrimator) with HCN absorbed in paper pulp, which is cut out in the form of thin discs. They use 2 ounces HCN per 1000 cubic feet of space.

Cyanogen chloride gas.—This gas has been used as a substitute for hydrocyanic acid gas. Its lachrymatory and irritant properties even in non-lethal concentration are efficient to warn of its presence. It does not injure foodstuffs, textiles, etc. For fumigation against rats the Public Health Service has employed 4 oz. sodium cyanide, 0.8 to 1.6 oz. sodium chlorate (exact amount varies with conditions not yet determined), 17 oz. commercial hydrochloric acid, and 17 oz. of water per 1000 cubic feet of space. The acid and water are mixed in barrels, crocks or buckets and the sacks containing the dry chemicals are dropped into the mixture by men wearing anti-cyanide gas masks. "Eggs" containing a mixture of sodium cyanide and sodium chlorate may be purchased and will obviate the hazard connected with mixing the loose chemicals.

### MISCELLANEOUS DISINFESTANTS

Pulicides.—For destruction of rat fleas one must accomplish the simultaneous destruction of rats by fumigation, bait poison, trapping, etc.

Among the liquid pulicides we have (1) crude petroleum (fuel oil) which is at times called Pesterine, (2) an emulsion of kerosene oil made as follows: Kerosene 20 parts, soft soap 1 part and water 5 parts. The soap is dissolved in the water by the aid of heat and the kerosene gradually stirred into the hot mixture.

As a general insecticide a mixture of 0.3 per cent pyrethrins (esters of the monovalent and divalent chrysanthemum acids) and 2.5 per cent B,B' thiocyanodiethyl ether (5 per cent Lethane 384, a trade name of Rohm & Haas for 50 per cent by volume of the aliphatic thiocyanate) in deodorized kerosene is a very efficient liquid insecticide (spray). As a supplement to the spray, a mixture in an inert carrier (sulphur, tale or clay) of pyrethrum powder and powdered derris or cube in such proportions as to give a content of 0.4 per cent pyrethrins and 1 per cent rotenone yields a highly successful insecticide powder.

Carboxide gas (a trade name of Union Carbide and Carbon Chemicals Corporation), which contains 10 per cent ethylene oxide in carbon dioxide, has been found useful as an insecticide when employed in the concentration of 6 pounds per 1000 cubic feet of space. The period of fumigation required is 3 hours (for a sealed space).

**Pediculicides.**—Owing to the great importance of lice in transmitting typhus fever, trench fever and relapsing fever their destruction is a vital consideration.

Although the body louse is the important transmitting agent, the head louse and possibly the crab louse should also be destroyed.

The subject of pediculosis has been much discussed on account of its importance among the troops in the European war.

For the destruction of head lice Pernet recommends:

- 1. Prevention: hair to be kept close cropped and clean.
- 2. For the nits: wipe them off with a solution of 1 in 30 phenol.
- 3. For the lice themselves: Unguentum hydrargyri ammoniati, diluted (gr.x to r oz.), or any fatty, sticky body well rubbed into the back of the head. Parassin lampoil (kerosene) also good, but not to be used near an open slame or light.

Blanchard considers camphorated alcohol or warm vinegar containing I to 1000 corrosive sublimate as useful for head lice. He also suggests the fumigation of clothes with tobacco as valuable for body lice.

For the destruction of body lice Pernet recommends:

- r. All body and bed-linen and clothes should be baked or sterilized by boiling.
- 2. Unguentum staphisagriae should be applied to neck-bands of vests and shirt in the region of the neck.

3. Alkaline baths to soothe the irritated skin.

Sublimed sulphur sprinkled in the bed and the clothes is very useful.

Castellani and Jackson have gone most extensively into the matter of louse destruction. Their conclusions are as follows: 1. In regard to solid and liquid insecticides, the substances which have been found to be deleterious to body lice are in the order of their efficiency: Kerosene oil, vaseline, guaiacol, anise preparations, iodoform, lysol, cyllin and similar preparations, phenol solution, naphthalene, camphor.

Pyrethrum has a very feeble action on lice, and boric acid, sulphur, corrosive sublimate, and zinc sulphate, when used in powder form, have apparently no action

whatever. As regards bedbugs, kerosene oil is the best insecticide. Next to it comes guaiacol, one of the most active drugs of those tried.

For ridding the body of lice the following steps are essential:

- 1. The hair of the body and head should be clipped.
- 2. The subject should be bathed, there being used freely kerosene-emulsion soap, prepared by boiling 1 part of soap in 4 parts of water, and then adding 2 parts of kerosene oil. The resultant jelly, when mixed with 4 parts of water, makes a liquid soap that is convenient to use and which may be applied effectively.
- 3. Following the bath, the body may be anointed with kerosene, special care being devoted to the hairy parts. Skin irritation may, however, require early removal of the oil.
- 4. It has been found that lice on clothing removed from the body may remain alive nine days and their eggs as long as forty days. The clothing, therefore, should be disinfested by one of the following methods.
- (a) Steam; (b) boiling for five minutes; (c) 5% compound cresol solution for 30 minutes; (d) fumigants such as sulphur dioxide and hydrocyanic acid gas. Chlorpicrin has also been employed.
- 5. In the absence of facilities for carrying out the steps described, or to prevent infestation subsequently, dusting powders are sometimes used. Of these the N.C.I. powder, containing commercial naphthalene, 96 Gm., creosote, 2 cc., and iodoform, 2 Gm., is the most widely known; but Moore's powder—creosote, r cc.; sulphur, o.5 Gm., and talc, 20 Gm.—is less irritating and is said to be six times as effective. It has also been recommended to wring out the underclothes in 5% compound cresol solution and then dry thoroughly, or to impregnate them with substances such as the halogenated phenols.

Raticides.—For large scale extermination of rats, especially during an outbreak of plague, fumigation with sulphur dioxide, hydrocyanic acid gas or cyanogen chloride gas is the most efficient method. For exterminating rats and in this way secondarily the rat fleas, besides the ordinary poisons such as As, P, etc., Rucker has recommended a poison composed of plaster of Paris 6 parts, pulverized sugar 1 part and flour 2 parts. This mixture should be exposed in dry place in open dishes. To attract the rats the edge of the dish may be smeared with the oil in which sardines have been packed. Phosphorus is the base of many of the commercial rat poisons. Its inflammable nature is a drawback. Furthermore rats soon learn to recognize its odor and refuse to eat food containing this poison. Barium carbonate is highly regarded as a rat poison, having neither taste nor odor. It is mixed with meal and bacon fat--about r part of barium carbonate to 4 parts of meal. In trapping rats one should frequently change the type of trap, and always allow the traps to be placed about the storchouse without being set for a day or so, to accustom the rat to its harmlessness. Again they should be handled with gloves so that they may not carry the odor of man. In the use of spring traps every effort should be made to disguise them by covering with saw dust. straw, chaff or meal. Bacterial vaccines, as the Danysz virus, are not considered satisfactory and are dangerous as possible causes of food poisoning in man.

Larvicides.—When drainage and filling of mosquito-breeding areas are not practicable and the use of fish not possible, various larvicidal agents may be used. The most common of these are petroleum, both crude and refined, Panama larvicide, (p. 583) crude phenol, cresol and a mixture of soft soap and petroleum. Trioxymethylene (paraformaldehyde) has been mentioned by Roubaud and others as being

efficient against anopheline larvae. The Marine Barracks at Quantico, Va., reported satisfactory results from the use of oiled sawdust in 1918. Barber and Hayne published an account of the use of Paris green mixed with inert dust to form a surface-deposit in 1921. The observation that Paris green is effective against the surface feeding anophelines but has no effect on culicine larvae has been confirmed by subsequent observers. One pound per acre is efficient, and in open water without vegetation much smaller quantities suffice. Airplanes equipped with hoppers lend themselves to thorough and economical distribution of larvicides. The Department of Agriculture tried dusting with Paris green from airplanes manned by Army pilots in 1923. Since that time airplanes have been used in various parts of the country to distribute Paris green and oiled sawdust. Oiled sawdust is probably the best general larvicide for use in this manner. Dry fine sawdust is impregnated with a mixture made from equal volumes of crank case oil and kerosene. It takes four to ten days to effect saturation depending upon the kind of sawdust used. Excess oil is permitted to drain from the impregnated sawdust before it is used.

**Destruction of Mosquitoes.**—Measures of protection against the immediate danger of infection from the adult mosquito merit attention. Screening, fumigation, repellents and swatting constitute the usual means.

Carter stated that wire screening with 16 meshes to the linear inch will exclude anophelines. Number 18 screen (18 meshes to the inch) was adopted on the Isthmus to exclude Aedes aegypti which will pass through number 16 screen. It is manifest that screening will not be effective unless particular attention is given to stopping up cracks around the doors, window screens and elsewhere. Holes through which pipes pass, drains, fireplaces not in use and even key holes should be sealed against mosquitoes. Canvas strips were found by von Ezdorf to be convenient in making window screens and screen doors tight. In quarters for temporary occupancy fine cloth netting nailed outside the windows and secured with battens will do. In infected areas where screening is not possible mosquito bars should be used on beds but must be properly applied to afford protection. They should be suspended so that they hang some distance over the mattress and inside the head and foot pieces of the bed so that the edges may be tucked in snugly under the mattress when one goes to bed. Howard states that the wearing of veils and gloves after sundown enforced at stations on the Italian railroads some years ago resulted in a great reduction of malaria.

The fumigants usually employed to destroy mosquitoes are dry sulphur dioxide, pyrethrum and Mim's culicide (phenol-camphor). The U. S. Public Health Service has also used hydrocyanic acid gas for this purpose.

Dry sulphur dioxide produced by burning 2 pounds of sulphur for every 1000 cubic feet is very effective if the spaces are made tight by stuffing or sealing all cracks and openings. Two hours exposure is sufficient. The vessels in which the sulphur is to be burned should rest on bricks or in a tub of sand to prevent fire but should not rest in water as is done in ordinary fumigation. It is said that in the absence of moisture sulphur dioxide causes no injury to household goods, fabrics or metals.

Pyrethrum is used in the proportion of 2 pounds to 1000 cubic feet. The powder is ignited, and after two hours exposure the mosquitoes must be swept up carefully and burned as pyrethrum, although it stuns the mosquitoes, cannot be depended on to kill. The expense is another deterrent to its use.

Mim's culicide is made by triturating equal parts of camphor and phenol. The resulting liquid is volatilized by gentle heat, 4 ounces being used for each 1000 cubic

feet. Goldberger states that, like pyrethrum, the fumes of this culicide stun but do not necessarily kill the mosquitoes. Care should be taken not to overheat this substance as the vapor is likely to catch fire. The lamp used to heat the container should stand in a vessel of water for this reason. In the absence of a convenient support, a piece of stovepipe is cut to form three legs. An alcohol lamp is inserted to heat a flat basin resting on the other end of the pipe.

Creel and Faget found that exposure for 15 minutes to the gas from 0.4 ounce of potassium cyanide per 1000 cubic feet of space was sufficient to kill mosquitoes. The amount for 1000 cubic feet yields "approximately 1 part cyanogen to 6000 parts of air, so dilute, in fact, as to practically eliminate all danger to human life. On repeated occasions we entered the fumigating room immediately upon opening the doors after mosquito fumigation without noticing any ill effects." In generating the hydrocyanic acid gas they used potassium cyanide, c.p., sulphuric acid (66B) and water combined in the proportions by weight of 1 part cyanide, 2 parts acid, and  $2\frac{1}{2}$  parts water.

For houses that cannot be screened properly by reason of their construction, Coogle found commercial creosote oil to be practical as a repellent for anopheline mosquitoes. The ceilings and walls of 25 houses in various sections of an anopheline-infested area were sprayed in the proportion of 1 gallon of creosote oil to 420 square feet. Anopheline mosquitoes were found in all the houses on several visits prior to the treatment. After spraying, no anopheline mosquitoes were found in any of these houses during three inspections at three-weeks intervals. Apparently the occupants did not object to the creosote oil and no ill effects were noted in any of those who slept in the rooms subsequent to the spraying.

Volatile oils, particularly citronella, pennyroyal, lavender and cedar, are commonly used on exposed parts of the body as repellents. Spirit of camphor, kerosene, oil of peppermint, oil of tar, lemon juice and vinegar have also been recommended. Samotz recommends dilution with 4 parts of liquid petrolatum to retard the evaporation of oil of citronella. Repellents to be applied to the body are poor substitutes for screening when we have to do with infective mosquitoes.

### COMMUNICABLE DISEASE CONTROL ON AIRCRAFT

Following the action of an International Convention for Aerial Navigation, at the Hague, in 1933, regulations concerning measures to prevent the introduction of communicable diseases have been established. As regards passengers, these follow the lines adopted for other modes of transportation. The communicable diseases which are the subject of special measures are: plague, cholera, yellow fever, exanthematous typhus, and small pox. In these regulations the period of incubation is reckoned as follows: 6 days for plague; 5 days for cholera; 6 days for yellow fever; 12 days for typhus; and 14 days for small pox. De-ratization and disinsectization measures are prescribed when indicated.

Mosquito Destruction.—Aircraft from tropical countries are required to take steps to destroy mosquitoes before arrival at a United States port. This is accomplished by spraying a culicide inside the aircraft compartments, which must not be toxic to human beings. Furthermore, it must be noninflammable.

The essentials of the spray at present advocated are one part of pyrethrum extract in oil and four parts of a light oil or carbon tetrachloride. The pyrethrum extract is such that one gallon of the extract contains the pyrethrins extracted from 20 pounds of standard pyrethrum flowers, the latter containing at least 0.9% of pyrethrins as

determined by chemical assay. The light oil is one approximating the characteristics of kerosene, and may vary within considerable limits provided the flash point is not too low.

The mixture may be made up in any proportions of extract, oil and carbon tetrachloride, providing only that the extract is at least 20%. For example, it may be any of the following: 1 part of extract with 4 parts of oil; 1 part extract, 3 parts oil and 1 part extract, 2 parts oil and 2 parts carbon tetrachloride; 1 part extract, 2 parts oil and 2 parts carbon tetrachloride; 1 part extract and 4 parts carbon tetrachloride. The last named is the mixture that has been specifically advocated by the Public Health Service, because it is to all intents and purposes completely noninflammable. Some objection has been raised to its use, however, because the carbon tetrachloride is slightly toxic. Whether it is to be used or not is not of particular moment from a quarantine standpoint, being a point which must be settled in accordance with the fire hazard.

Any of these mixtures when sprayed through the air in the form of very fine droplets in the amount of 5 cc. per thousand cubic feet of space will fatally poison mosquitoes within 5 to 10 minutes.

The action of the pyrethrins on mosquitoes, and probably on other insects, is due apparently to direct absorption following actual wetting of the surface by the pyrethrins in solution. It is for this reason that it is essential that at least 20% of the mixture be an oil that does not immediately evaporate. If 100% carbon tetrachloride is used with the same pyrethrin content, the lethal effect is much less, presumably due to the rapid evaporation of the carbon tetrachloride and the consequent precipitation of the pyrethrins in solid form.

There has been objection made to the use of carbon tertachloride by some of the aviation executives. Furthermore, it may be that a combination of pyrethrin and rotenone is more efficient and more culicidal than pyrethrin alone. Rotenone is a white crystalline compound obtained from derris and other plant sources. The question of the best culicide for aircraft is being actively studied, and the final selection is to be awaited

### J. ANATOMICAL AND PHYSIOLOGICAL NORMALS

In examinations in the pathological or chemical laboratory the following may be considered approximately as normal findings:

1. Anatomical Normals. Averages.

Adrenals. Length, 2.4-2.8 inches (6-7 cm.). Breadth, 1.2-1.4 inches (3-3.5 cm.). Weight, 0.17-0.21 ounce (5-6 grams) each. Left usually larger.

Aorta. Length, varies, 17-20 inches (42.5-50 cm.). Thickness of wall, 0.06-0.08 inch (1.5-2 mm.). Diameter, 0.75-1.25 inches (1.7-3 cm.). Weight, 1.2-1.6 ounces (35-45 grams).

Appendix.—Length, quite variable, 3.5-4 inches (9-10 cm.). Diameter 0.25 inch (6 mm.). Weight, 0.25-0.5 ounce (7-14 grams).

Bladder. Capacity, 16 ounces (500 cc.) when normally distended. Thickness of wall, 0.1 inch (2.5 mm.). Weight, r-2.1 ounces (30-60 grams).

Brain. Weight, female 44-45 ounces (1250-1275 grams), male 48-51 ounces 1365-1450 grams). Length, 6.5 inches (16.5 cm.). Transverse diameter, 5.5 inches (14 cm.). Vertical diameter, 5 inches (12.7 cm.). Dimensions in female being 0.4 inch (12 cm.) less.

Fallopian tubes. Length, 3-5 inches (7.6-12.6 cm.). The right usually the longer. Diameter of lumen averages 0.1 inch (2.5 mm.).

Gall bladder. Length, 3-4 inches (7.5-10 cm.). Diameter, 1-1.25 inches (2.5-3 cm.). Thickness of wall, 0.04-0.07 inch (1-2 mm.). Capacity, 1-1.5 ounces (30-45 cc.).

Heart. Weight, female 8.8–9.8 ounces (250–280 grams), male 9.5–12.7 ounces (270–360 grams). Length, 4.5–5.5 inches (11.5–14 cm.). Breadth, 3–4 inches (7.5–10 cm.). Thickness, 2–3.1 inches (5–8 cm.): Thickness, wall left ventricle, 0.35–0.47 inch (9–12 mm.), right ventricle, 0.1–0.12 inch (2.5–3 mm.). Circumference, mittal orifice, 4.1–4.3 inches (10.4–10.9 cm.). Circumference, tricuspid orifice, 4.7–5 inches (12–12.7 cm.). Circumference, aortic orifice, 3–3.2 inches (7.7–8 cm.). Circumference, pulmonary orifice, 3.4–3.6 inches (8.5–9 cm.).

Intestines. Small intestine, length, 22.5 ft. (6.75 meters); 26 jejunum and 36 ileum. Diameter from 1.85 inches (47 mm.) in duodenum to 1.06 inches (27 mm.) at the end of ileum. Large intestine, length, 70.9-76.8 inches (180-195 cm.). Duodenum, length 10.2-11.2 inches (26-28.5 cm.).

Kidneys. Weight, left, 5.3 ounces (150 grams), right, 5 ounces (140 grams). Thickness of cortex, 0.4 inch (1 cm.). Length, 4.5 inches (11.5 cm.). Breadth, 2.50 inches (6.2 cm.). Thickness, 1.25 inches (3.2 cm.). The left longer and the right thicker.

Liver. Weight 50-60 ounces (1440-1680 grams). Greatest transverse diameter, 7.9-9.5 inches (20-24 cm.). Greatest antero-posterior diameter, 3.9-5.9 inches (10-15 cm.). Vertical diameter, 5-6 inches (12.7-15 cm.).

Lungs. Weight, combined, 36-45 ounces (1020-1290 grams). Weight, male, right lung, 24 ounces (680 grams), left lung, 21 ounces (600 grams). Weight, female, right lung, 17 ounces (480 grams), left lung, 14.8 ounces (420 grams). Length, 10-12 inches (26-30 cm.). Antero-posterior diameter at base, 7-8 inches (17.5-20 cm.). Transverse diameter at base 4-5 inches (10-12.7 cm.). The right lung is shorter, broader and thicker than the left. Dimensions in the female average 1 inch (2.5 cm.) less.

Mammary gland. Weight in adult, 5.25-7 ounces (150-200 grams). Weight during lactation, 14-31.75 ounces (400-900 grams).

Ocsophagus. Length, 10-12 inches (25-30 cm.). Diameter of lumen, 1.25 inches (3 cm.). Thickness of wall, 0.3 inch (8 mm.). Weight, 1.4 ounces (40 grams).

Ovaries. Weight (each), 0.12-0.25 ounce (4-8 grams). Length, 1.5 inches (3.8 cm.). Breadth, 0.75 inch (1.9 cm.). Thickness, 0.5 inch (1.2 cm.).

Pancreas. Weight, quite variable, 2.1-4.8 ounces (60-135 grams). Length varies, average 6-8 inches (15-20 cm.).

Parathyroids. Length, 0.2-0.25 inch (6-7 mm.). Breadth, 0.15-0.17 inch (3-4 mm.). Thickness, 0.05-0.075 inch (1.5-2 mm.).

Pincal gland. Length, 0.4 inch (1 cm.). Breadth, 0.2 inch (5 mm.). Thickness, 0.2 inch (5 mm.). Weight, 3 grains (0.2 gram).

Pituitary body. Length, 0.3 inch (8 mm.). Breadth, 0.5 inch (1.2 cm.). Weight, 5-10 grains (0.3-0.6 gram).

Prostate. Weight, 0.8 ounce (22 grams). Length, 1.25-1.5 inches (3.1-3.8 cm.). Breadth, 1.5-1.75 inches (3.8-4.5 cm.). Thickness, 1 inch (2.5 cm.).

Salivary glands. Parotid, weight, 0.8-1 ounce (25-30 grams). Sublingual, weight, 0.06-0.09 ounce (2-3 grams). Submaxillary, weight, 0.25-0.3 ounce (8-9 grams). Seminal vesicles. Length, 2 inches (5 cm.).

Spinal cord. Length, 18 inches (45 cm.). Weight, 0.9-1 ounce (27-30 grams). Transverse diameter averages 0.5 inch (1.2 cm.). Antero-posterior diameter averages 0.4 inch (9 mm.).

Spleen. Weight, 5.5-6.9 ounces (155-195 grams). Length, 4-5 inches (10-12.5 cm.). Breadth, 3 inches (7.7 cm.). Thickness, 1-1.5 inches (2.5-3.7 cm.).

Stomach. Capacity, 1-2 quarts (1-2 liters). Thickness of wall, 0.25 inch (6 mm.). Weight, 4.5-6.2 ounces (125-175 grams).

Testes. Weight, 0.65-0.8 ounce (20-25 grams) each. Length, 1.5 inches (3.8 cm.). Breadth, 1 inch (2.5 cm.). Thickness, 0.8 inch (2 cm.).

Thoracic duct. Length, 15-18 inches (37.5-45 cm.).

Thymus gland. Weight at birth, 0.5 ounce (13.7 grams) and increases to 0.9 ounce (26.2 grams) at end of second year when it gradually decreases until gland disappears. Dimensions at birth, length, 2.4 inches (6 cm.), breadth, 1.5 inches (3.7 cm.) and thickness 0.25 inch (6 mm.).

Thyroid. Transverse diameter, 2.4-2.8 inches (6-7 cm.). Height, 1.2 inches (3 cm.). Weight, 1-1.4 ounces (30-40 Gm.).

Ureters. Length, 11.2-12 inches (28-30 cm.). Slightly longer on left side and longer in male. Diameter of lumen varies, averages 0.1 inch (2.5 mm.).

Urethra. Male. Length, 6.4-8.25 inches (16-20.6 cm.). Prostatic, 1-1.25 inches (2.5-3.1 cm.); membranous, 0.6-1 inch (1.5-2.5 cm.) and the anterior 4.75-6 inches (12-15 cm.). Female. Length, 1.5 inches (3.8 cm.). Diameter of lumen averages 0.25-0.4 inch (7-10 mm.).

Uterus. (Virginal) length, 2.8 inches (7 cm.). Breadth, 1.6 inches (4 cm.). Thickness, 1 inch (2.5 cm.). Weight, 1.4-1.8 ounces (40-50 Gm.). The dimensions of a multiparous uterus are each increased 1 cm. or more and the weight is increased 0.7 ounce (20 grams). Length of cavity in virgin, 2 inches (5 cm.), in multiparae, 2.25 inches (5.7 cm.).

Vagina. Length, 3-3.5 inches (7.6-8.9 cm.). The posterior wall is slightly longer than the anterior.

II. PHYSIOLOGICAL NORMALS (ADULT).

Blood: (Values are in mg. per 100 cc. whole blood unless otherwise noted.)

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Specific gravity...... 1.041 to 1.067 (1.026 to 1.032 for serum)
Reaction (see p. 682)..... pH 7.3 to 7.5
Serum albumin..... 3.8 to 5.2%
Amino-acid nitrogen...... 6 to 8 (plasma, 4 to 7)
Ammonia nitrogen..... about o.1
Uric acid (Folin-Wu method)...... 2 to 3 (extremes, 1 to 4)
"Creatinine"..... 1 to 2
Creatine..... 3 to 5 (plasma, o to 3.8)
Sugar (Folin-Wu method)...... 70 to 120 (60-100 true)
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Fat (Bloor's fat method)..... about 600
Lecithin (Bloor's "lecithin")...... 30 (plasma, 22)
Acetone bodies..... o to 4
Bicarbonate (plasma) . . . . . . . . . . . . 53 to 75 vol. % CO<sub>2</sub>
Oxygen capacity...... 20.0 vol. %
CO2 tension (arterial)..... about 40 mm. Hg.
Phosphorus, total (as H<sub>3</sub>PO<sub>4</sub>)..... about 120 (plasma 35 to 40)
Phosphates, inorganic (as P)..... (serum) 3.2 to 4.3
Sulphates (as S)..... 0.5 to 1.0
Cerebrospinal fluid (see p. 627): (Values are in mg. per 100 cc. unless otherwise
noted.)
Specific gravity..... 1.001 to 1.008
Pressure..... 5-12 mm. Hg. or 70-160 mm. H<sub>2</sub>O
Serum albumin about 6
Serum globulin ..... 20-30
Nonprotein nitrogen...... 15-35
Creatinine..... 1-2
Sugar..... 50-80
Chlorides..... 720-750
 Stomach contents (see normal acidity curve, p. 755):
 One hour after Ewald test meal:
Reaction . . . . . . . . . . . . pH o.q-1.6
Quantity..... 40 to 50 cc.
Total acidity..... 40 to 80°
Free hydrochloric acid...... 25 to 50°
Residuum (fasting):
Quantity..... 20 to 100 cc. (rarely 150)
Total acidity..... 10 to 50°
Free acidity..... o to 30°
Pepsin..... 3 (Mett)
Bile..... present in about 60% of cases
Gastric mucus..... traces
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Duodenal contents (see pp. 759, 760).

Urine (24-hour specimen; values in Gm. unless otherwise specified):

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Quantity.....
                       25 cc. per kg. body weight
Specific gravity ....
                       1.015 to 1.025
Reaction.....
                      acid to litmus (pH 4.8 to 8.0, average about 6.0)
Total solids .....
                       55 to 70
Total nitrogen....
                       10 to 16
Urea
                       10 to 40 (supplies about 85% of total N)
Uric acid.....
                      0.2 to 2
Creatinine....
                       1 to 1.5
Ammonia.....
                      0.5 to 1.2 (see NH3 quotient, p. 730)
Hippuric acid.....
                       0.6 to 1
Albumin (see p. 716)
Chlorides (as NaCl)...
                       10 to 15
Sulphur (as H<sub>2</sub>SO<sub>4</sub>)...
                       2 to 2.5
Phosphorus (as P<sub>2</sub>O<sub>5</sub>).
                       I to 5 (about 90% of phosphates are acid phosphates)
Calcium (as CaO).....
                       o.r to o.6
Magnesium (as MgO).
                       0.2 to 5.6
Acetone bodies.....
                     about 10 mg.
Sugar (see p. 721)
  Faeces:
Average daily output, moist faeces (Hawk) . . . . 110 Gm.
24-hour amount on ordinary mixed diet...... 110 to 170 Gm. (25 to 45 Gm. solids)
24-hour amount on vegetable diet..... to 350 Gm. (about 75 Gm. solids)
  Fatty substances (averages expressed as per 1 Gm. dried faeces):
Respiration:
    Alveolar air:
Carbon dioxide...... about 5.5% (35 to 40 mm. CO<sub>2</sub> tension)
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Air hunger in diabetes or chronic nephritis begins only when  ${\rm CO_2}$  tension has fallen to 20 or 25 mm.

Nitrogen..... 80%

### K. IMPORTANT DISEASES AND INJURIES DUE TO PLANTS

Arrow Poisons.—Many primitive people add to the wound effect of their arrows, that of poison. Although animal secretions, particularly snake venoms, are used, they are inferior in effect to the vegetable poisons. In some arrow poisons snake venom is combined with the plant extract, but as the users often treat the snake venom in such a way as to destroy the toxins, the effect must be due entirely to the vegetable poison. Material of bacterial nature is also used, particularly the tetanus bacillus, but most of the potent arrow poisons are vegetal.

Acceanthera.—An extract is prepared from the wood or roots of various species of the genus, and is probably the most widely used poison among the African natives.

The action is powerful and rapid, stopping the heart in a few minutes. The active principle is ouabain, a glucoside related to those in digitalis.

Strophanthus.—Various species of this genus are used by African tribes, extracts being prepared from the seeds and often mixed with heads of snakes. It has the digitalis effect.

Strychnos.—The principal arrow poison of the Amazon river tribes is curara, made by extracting the bark of various Strychnos species. The curara is supposed to contain curarine chiefly, but there are other alkaloids present. The alkaloid arrests voluntary movements, by interrupting connection between the peripheral nerves and the muscles. The animal lies helpless on the ground. Certain Malay tribes use poisons from this genus, but their arrow poisons seem to contain strychnine and brucine as well as curarine, so that in animals wounded by such poisoned darts we have convulsive phenomena, as well as the action on the motor end plates. Other poisons used by certain Himalayan tribes seem to have an aconite basis.

Atriplicism.—A disease of North China supposed to be due to the toxic properties of a weed, Atriplex littoralis, which grows in gardens around Pekin, but possibly caused by a small insect often found on the weed, as it is claimed the weed will not cause disease if well washed before eating. It is only in times of famine that the weed is eaten, and then only by the very poor. About 15 hours after ingestion there appears itching of the fingers, quickly followed by swelling and discoloration. This swelling extends up the backs of the hands and outer surface of the forearms. The face also becomes swollen so that the eyelids may be closed, and the nose becomes cyanosed and cold. The swollen parts may ultimately develop blisters and ulcers.

Cannabis Indica.—This is an important drug addiction in Central Asia and the plant is generally called Indian hemp. In India the forms of the drug generally used are "gangah," the dried flowering tops, which is smoked, mixed with tobacco; "bhang," a mixture of the dried leaves and capsule, which is made into decoctions and is the cheapest form of the drug; and "charas," the resinous exudate obtained from the cut female heads of the plant. This is the most expensive and most concentrated of the preparations. In Arabia a confection is made from charas and is known as "hashish." The drug gives a feeling of well being followed by hallucinations of sight and hearing—often of sensual character. This is followed by dimness of vision, drowsiness and stupor. Addicts may become insane.

Favism.—This is a disease caused by inhaling pollen from the flowers of the bean plant or by eating the bean (Vicia faba)—most frequent in Italy and especially southern Sicily. Heredity seems to play a part, some families giving a history of favism over many generations. (Idiosyncrasy.) Ingestion of raw beans is more apt to cause it than eating cooked beans. About half of the cases are due to exposure to blooming plants. There seems to be no relation between the amount of bean caten and the severity of the symptoms. This, taken in connection with the hypersensitiveness to the bloom, would surely indicate some form of allergy. About 8% of the cases are fatal. McCrae and Ullery (1933) reported a case in Philadelphia. The patient, an Italian, gave a history of attacks from pea blooms when living in Sicily. Following a hearty meal of beans he began to feel very ill and two hours.later on voiding urine, noticed that it was black. He was of the opinion that the beans he had eaten were from Sicily. Five days later he was admitted to hospital showing ashen grey color of facies and pallor of mucous membranes which was accompanied by jaundice. There was some fever and the urine was absolutely black (haemoglobinuria) and showed many granular casts.

Red cells were about 1,400,000 and the haemoglobin 38%. The leukocyte count was 19,300 with 73% polymorphonuclears. Reticulocytes were about 14%. There was about 11% of erythroblasts and rather marked anisocytosis. Blood urea nitrogen was 37. Paroxysmal haemoglobinaemia was excluded by the Donath-Landsteiner test. (The patient had a positive Wassermann.) A skin test showed allergy to an extract of the beans eaten. This case was apparently the first to be reported in English or American literature. On account of the great number of Italians living in the United States it must be that the allergy described above is not infrequent. In Italy it is noted that the attack comes on shortly after exposure with an irregular fever and haemoglobinuria. The sudden and great fall of red cells may cause death in a very short time. When the patient does not die recovery takes place quite satisfactorily. Epinephrin and blood transfusions are to be considered.

Kava.—An intoxicating (non-alcoholic) drink is made from the roots or leaves of the pepper plant, *Piper methysticum*, and is a ceremonial beverage in many of the islands of the South Pacific. The parts of the plant are first chewed by young girls who have good teeth and good health, and the masticated material is put in a bowl and treated with coconut milk. A sort of quiet, drowsy intoxication, with weakness of the legs, results in those not habituated. The active principle seems to be a glucoside, and it is supposed that it is broken up by a salivary ferment, to produce a substance responsible for the intoxication.

Lathyrism.—A form of spastic paraplegia due to the eating, in times of great scarcity, of bread made from the flour of the chick-pea, Lathyrus sativus, or other species of vetches. The disease comes on insidiously with pains in the back and weakness of the legs. As the affection develops, the legs are dragged along with great effort, and there is a tendency to fall. Wasting of the leg muscles is common. Reflexes are exaggerated. Incontinence of urine and loss of sexual power are important symptoms. Upper extremities are only rarely involved. There is no mental or cardiac involvement. The disease runs a very chronic course but is rarely fatal. A deficiency in vitamin A has been suggested as a factor in the production of the disease.

Milk Sickness.—Cattle eating richweed (white snakeroot, Eupatorium ageratoides) in Eastern United States, or rayless goldenrod in Texas, acquire a serious disease called trembles. Man ingesting the milk or butter from a cow so affected suffers from an illness more or less serious according to the amount of toxic material eliminated in the milk. In cattle, the symptoms consist mainly of weakness and uncertain gait, together with clonic muscular contractions which give rise to the descriptive name trembles. In man there are observed anorexia, nausea, and vomiting, which prevent the taking of food and water, and soon bring about an acidosis characterized by a high mortality. The later symptoms are subnormal temperature, extremely low blood pressure, presence of diacetic acid in the urine, and of the odor of acetone in the breath and urine. The blood shows a marked ketosis, a lipaemia and a hypoglycaemia. Treatment should be directed toward restoring the acid-base equilibrium.

Mushroom Poisoning.—The best known edible mushrooms are (in England and the United States) the meadow mushroom, Agaricus campester, which grows only in open pastures and (in France and Italy) the champignon, Marasmius orcades. There are no criteria by means of which edible mushrooms can be distinguished from poisonous varieties. There is a wide-spread, entirely erroneous belief that if a silver coin is put in the dish in which mushrooms are cooked, it will be tarnished if poisonous varieties are present. They can not be recognized by the taste—the most poisonous species are

said to have a very agreeable flavor. In Washington an Italian officer who was regarded as an expert in the recognition of edible species, purchased in a market some mushrooms which had been collected in nearby Virginia. He breakfasted on these mushrooms and spoke of their fine flavor. In about 15 minutes he became acutely ill, developed blindness, dysphagia and convulsions and died within 24 hours. If one has not the expert knowledge required to identify the species with certainty, one should cut only mushrooms which have been passed upon by a competent (preferably official) inspection. Even in cultivated beds, poisonous species occasionally develop.

There is marked individual variation in susceptibility. In the case of a family of 6 poisoned by Amanila nappa, reported by Bentkowski, four died (one of whom had eaten only a mouthful); one became ill but recovered, and the sixth who had eaten heartily suffered no ill effects.

In the United States most cases of poisoning are due either to Amanita musicaria, the fly amanita, or to A. phalloides, the death cup. Both species are widely distributed and very common. One feature which helps to identify this genus is the persistence of a portion of the veil, encircling the stem a little below the cap.

The poison in A. muscaria is muscarin, an alkaloid related to pilocarpin. This type is distinguished by the early appearance of symptoms (within three hours), and death in the fatal cases occurs within 24 hours. There are nausea, vomiting, diarrhoea, severe abdominal pain, sweating, salivation, lachrymation, miosis, often a slow irregular pulse, and in fatal cases, convulsions and coma. Atropin is an efficient antidote, and although the symptoms are violent, the mortality is low (about 10%) in properly treated cases.

The poison in A. phalloides is a toxin. In this type the symptoms are late in appearing (6 to 18 hours), and although the mortality is from 50% to 70%, death usually occurs only after 5 to 8 days. In addition to the gastrointestinal symptoms noted above, there may be great thirst, anuria, jaundice (after two or three days), cyanosis, drousiness, delirium or coma. There is degeneration of the renal tubular epithelium and liver necrosis; and extensive degeneration of the ganglion cells of the cerebral cortex, basal ganglia, cerebellum and brain stem have been described (Vander Veer and Farley, 1935). Atropin has no effect. An antitoxin has been used in Europe with alleged good results.

Another rarer type of mushroom poisoning, resulting in an acute haemolytic anacmia, haemoglobinuria and jaundice, has been described. Recovery is the rule.

Opium.—The use of opium is common among the inhabitants of India and the Orient, not to refer to the drug addicts of Europe and America. In India opium is almost invariably taken in pill form and with its use in this way the ill effects are much reduced. The mental, moral and physical deterioration so common in those who smoke opium, as in China and Persia, or who use morphine or other alkaloids hypodermically, is not so marked. There is also less tendency to increase the dose. Every medical man should read DeQuincey's "Confessions of an English Opium Eater," to appreciate the slight effect this habit had on the author for the first few years of his addiction. He took the drug as did the native of India.

Plants Causing Dermatitis Venenata.—There are a great many plants, in various parts of the world, which cause various types of dermatitis—erythematous, vesicular or urticarial. The best known of these plants belong to the *Rhus* family. In the United States poison oak and poison ivy are frequent causes of skin eruptions, but it is necessary that contact be made with the plant—the idea that a volatile principle is given off is not

Vomiting Sickness of Jamaica.—This, a disease of the West Indies, is due to eating unripe "ackee," the local name of the fruit of Blighia sapida. The fully ripe, opened fruit is harmless and is a valued food. Children, eating the unripe fruit, the arilli of which contain the poison, become suddenly ill in about 2 hours with abdominal pain and vomiting. There is a period of apparent recovery lasting a few hours after which vomiting again begins, and almost coincidently convulsions and coma, ending generally in death.

Miscellaneous Sources of Plant Poisoning.—In addition to the diseases described, numerous other instances of poisoning by plants are known, some accidental in origin, and some intentional.

Chestnut, however, states that in the United States there are only about 30 species of plants ever associated with accidental poisoning in man, poisoning with them generally resulting from confusing poisonous plants and harmless ones, as mistaking water hemlock for edible roots. Prussic acid occurs in many valuable foods, and, if not removed, may produce serious results and even death. Bitter cassava, for example, contains a glucoside which in the presence of water sets free HCN; and, in order to avoid poisoning, the tuber must be scraped, grated, squeezed free of its milky juice, and then thoroughly washed. Oxalic acid likewise is contained in variable quantity in many edible plants, as sour grass and rhubarb, and may cause poisoning if ingested in sufficient quantity. Ergotism is another well-known illustration of accidental poisoning from the ingestion of food plants normally wholesome.

Various species of Jatropha (physic nuts) are found in India and the West Indies and symptoms of severe gastrointestinal irritation, more or less like that of croton oil, result from eating the seeds. The leaves of Jatropha urens have irritating hairs which may cause swelling of the lips, nausea and prostration. In the Philippines cases of poisoning by the fruit of Illicium religiosum, which is used to make a drink called "sanki," show symptoms resembling features of cholera, strychnine poisoning or even meningitis. In the West Indies a fruit resembling an apple grows on the "manchineel tree" (Hippomane mancinella) and when eaten gives rise to swelling of the lips and blisters and erosions of the buccal mucosa. Nausea, vomiting and difficulty in swallowing may be present and more rarely manifestations of profound collapse. The root of Gloriosa superba, a plant widely distributed in tropical Asia and Africa, has been used to produce poisoning. It is sometimes called wild aconite, and the physiological effects are

those of aconite poisoning—tingling and numbness of lips and pharynx, nausea, vomiting, abdominal pains, cardiac depression and collapse symptoms. Consciousness is retained until the late stages when convulsions may appear. Various plants belonging to the order Solanaceae are used in many parts of the tropical world, and by criminals in temperate climates, to produce unconsciousness. The seed of Datura fastuosa was used by the Thugs in India to produce unconsciousness, and various other plants, whose alkaloids have the belladonna action, are used by the natives of many parts of the tropical world. The dried leaves of Hyoscyamus niger, or henbane, is the basis of some of the "knock out" drops used by the underworld. In poisoning from these plants we have a flushed face, widely dilated pupils, with the eyes bright and shining. The throat is very dry. There is marked disturbance of vision. At first the victim is very talkative and soon becomes violent, but later on drowsiness sets in followed by coma. The yellow oleander, Cerbera thevetia, is a very poisonous plant found in India. The poisonous principle is a glucoside, which is found in the milky juice.

### L. COMMUNICABLE DISEASES

One of the most useful guides to the control of communicable diseases is the 1935 report of the Committee of the American Public Health Association. In this publication, which is for sale by the Superintendent of Documents, Washington, D. C. (price, five cents), will be found very complete directions for the various procedures required for public health management of each of the following diseases:

### List of Communicable Diseases for Which Notification Is Usually Required in the States and Cities of the United States

Actinomycosis. Ancylostomiasis (hookworm disease).

Anthrax.

Chicken pox (varicella).

Conjunctivitis, acute infectious.

Dengue. Diphtheria.

Dysentery, amebic (amebiasis).

Dysentery, bacillary.

Encephalitis, infectious, lethargic and

nonlethargic.

German measles (rubella).

Glanders (farcy). Gonorrhea.

Influenza. Leprosy.

Malaria.

Measles (rubeola).

Meningococcus meningitis.

Mumps (parotitis). Paratyphoid fever.

Plague, bubonic, septicemic, pneumonic.

Pneumonia, acute lobar.

Poliomyelitis. Psittacosis.

Puerperal infection (puerperal septi-

cemia). Rabies.

Rocky Mountain spotted (or tick) fever.

Scarlet fever (scarlatina).

Septic sore throat (streptococcus throat

infection).

Smallpox (variola). Syphilis.

Tetanus. Trachoma. Trichinosis.

Tuberculosis, pulmonary.

Tuberculosis, other than pulmonary.

Tularaemia. Typhoid fever. Typhus fever.

Undulant fever (brucellosis).

Whooping cough.

Yellow fever.

### SUPPLEMENTARY LISTS

В

Communicable Diseases or Infestations Occurring in the United States and Insular Possessions, but for Which Notification to the Health Authorities Is Not Everywhere Required

Ascariasis. Pediculosis.

Common cold. Rat-bite fever (sodoku).

Coccidioidal granuloma. Relapsing fever. Filariasis. Ringworm.

Icterohemorrhagic jaundice (Weil's Scabies.

disease). Schistosomiasis.

Impetigo contagiosa. Vincent's infection (angina, stomatitis).

Lymphogranuloma venereum (inguinale) Yaws.

and climatic bubo.1

Diseases of Concern to Health Officers Because of Their Group or Epidemic Occurrence and the Practicability of Their Prevention, and for These Reasons Often Included among Those Notifiable to the Health Authority, but Not to Be Considered Communicable in the Usual Sense of the Term

Botulism.

Pellagra.

Food infections and poisonings.

The committee adopted the following definitions of terms:

- r. Carrier.—A person who, without symptoms of a communicable disease, harbors and disseminates the specific micro-organisms. As distinct from a carrier, the term "infected person" is used to mean a person in whose tissues the etiological agent of a communicable disease is lodged and produces symptoms.
- 2. Cleaning.—This term signifies the removal by scrubbing and washing, as with hot water, soap, and washing soda, of organic matter on which and in which bacteria may find favorable conditions for prolonging life and virulence; also the removal by the same means of bacteria adherent to surfaces.
- 3. Contact.—A "contact" is any person or animal known to have been sufficiently near an infected person or animal to have been presumably exposed to transfer of infectious material directly, or by articles freshly soiled with such material.
- 4. *Delousing*.—By delousing is meant the process by which a person and his personal apparel are treated so that neither the adults nor the eggs of *Pediculus corporis* or *Pediculus capitis* survive.
- 5. Disinfection.—By this is meant the destroying of the vitality of pathogenic micro-organisms by chemical or physical means.

When the word "concurrent" is used as qualifying disinfection, it indicates the application of disinfection immediately after the discharge of infectious material from the body of an infected person, or after the soiling of articles with such infectious discharges, all personal contacts with such discharges or articles being prevented prior to their disinfection.

<sup>1</sup> This title does not include granuloma venereum (inguinale), which is a different clinical condition.

When the word "terminal" is used as qualifying disinfection, it indicates the process of rendering the personal clothing and immediate physical environment of the patient free from the possibility of conveying the infection to others, at the time when the patient is no longer a source of infection.

- 6. Disinfesting.—By disinfesting is meant any process, such as the use of dry or moist heat, gaseous agents, poisoned food, trapping, etc., by which insects and animals known to be capable of conveying or transmitting infection may be destroyed.
- 7. Education in personal cleantiness.—This phrase is intended to include all the various means available to impress upon all members of the community, young and old, and especially when communicable disease is prevalent or during epidemics, by spoken and printed word, and by illustration and suggestion, the necessity of:
  - (1) Keeping the body clean by sufficiently frequent soap and water baths.
- (2) Washing hands in soap and water after voiding bowels or bladder and always before eating.
- (3) Keeping hands and unclean articles, or articles which have been used for toilet purposes by others, away from mouth, nose, eyes, ears, and genitalia.
- (4) Avoiding the use of common or unclean eating, drinking, or toilet articles of any kind, such as towels, handkerchiefs, hairbrushes, drinking cups, pipes, etc.
- (5) Avoiding close exposure of persons to spray from the nose and mouth, as in coughing, sneezing, laughing, or talking.
- 8. Fumigation.—By fumigation is meant a process by which the destruction of insects, as mosquitoes and body lice, and animals, as rats, is accomplished by the employment of gaseous agents.
- 9. Isolation.\(^1\)—By isolation is meant the separating of persons suffering from a communicable disease, or carriers of the infecting micro-organism, from other persons, in such places and under such conditions as will prevent the direct or indirect conveyance of the infectious agent to susceptible persons.
- ro. Quarantine. —By quarantine is meant the limitation of freedom of movement of persons or animals who have been exposed to communicable disease for a period of time equal to the longest usual incubation period of the disease to which they have been exposed.

It is still considered necessary to require strict isolation of the patient for the period of communicability, and quarantine or immunization of contacts in certain diseases, notably smallpox. However, in some other diseases, such as poliomyelitis and encephalitis, isolation of the patient has but little apparent effect in limiting the spread of the disease, and the period of communicability is not known with reasonable accuracy in any given case.

Case-to-case infection is relatively infrequent in these latter two diseases; and yet the patient must be regarded as a potential source of infection and suitable precautions must be taken, even if these barriers to transmission of the disease are but partially effective. Uncertainty as to the exact duration of the period of communicability does not justify neglect of reasonable isolation measures but rather adds to our obligation to

<sup>1</sup> In view of the various ambiguous and inaccurate uses to which the words "isolation" and "quarantine" are not infrequently put, it has seemed best to adopt arbitrarily the word "isolation" as describing the limitation put upon the movements of the known sick or "carrier" individual or animal, and the word "quarantine" as describing the limitations put upon exposed or "contact" individuals.

educate patients, the family, and the attending physician in the advantages to be had from separating the sick from the well, and in taking precautionary measures voluntarily when the presence of a communicable disease is suspected and before a diagnosis is established, after the official period of isolation is past, and generally during the epidemic prevalence of such diseases in the community.

The five specific objectives of personal cleanliness as defined above (7), if conscientiously attempted, will materially aid in reducing the amount and frequency of infection.

Isolation of a communicable disease from visitors is often of benefit to the patient as well as a protection to others; quiet, freedom from the excitement and fatigue of visits, and complete rest are important factors in the medical and nursing management of such patients and directly contribute to recovery.

- 11. Renovation.—By renovation is meant, in addition to cleansing, such treatment of the walls, floors, and ceilings of rooms or houses as may be necessary to place the premises in a satisfactory sanitary condition.
- 12. Report of a disease.—By report of a disease is meant the notification to the Health Department and, in the case of communicable disease in animals, also to the respective Department of Agriculture which has immediate jurisdiction, that a case of communicable disease exists or is suspected of existing in a specified person or animal at a given address.
- 13. Susceptible.—A "susceptible" is a person or animal who is not known to have become immune to the particular disease in question by natural or artificial process.
- 14. Virus, filterable.—The term "filterable virus" as defining the etiological agent of certain diseases is used in the sense of a causal agent differentiated from other kinds of infectious agents such as bacteria, protozoa, etc. Many of these filterable viruses can be grown in vitro in the presence of living susceptible cells, and such cultures will produce regularly typical diseases in animals and in man. The term "filterable virus" has a significance comparable to that of bacterium, spirochete, or protozoon. The term "filterable virus" is as definite a description of an etiological agent as is the statement that the typhoid bacillus causes typhoid fever. The idea conveyed by the statement that a filterable virus is the etiological agent is that the cause of this disease is known, even though present knowledge does not permit further precision in distinguishing among filterable viruses except by reference to the name of the disease produced by each.

From this source, and other more recent ones, we have compiled the following table of the more common communicable diseases.

# COMMON COMMUNICABLE DISEASES Based on Report of Committee of A. P. H. Association

	Methods of control and remarks	lisolation of case or cariers who have come in close contact with infected individuals until pharayega and cultures heart one week after at least one week after ferry subsides even with negative cultures. Vaccine in munication doubtful value. Polyvalent artisenum necessitated as it atkes time to identify type. Carriers arrived develop disease. Prevent overcrowding and deficient ventilation.	No immunization. Bx- clude patient from solvol and contact with sus- ceptible children. Virus less resistant than that of variola.	Isolate case or carrier until one obtains two negative cultures from nose and intoot at least 2 days apart. After lesions dis- appear in 2 weeks- rarely they persist 2 to 6 months. Carry out Schick tests on contacts and nurses. Exposed susceptibles that cannot be beyt under daily ob-
issociation	Salient clinical features	Rapid onset with marked headache, stiffness of neck and vomiting. Cutaneous hyperaestlesia. Often Signs. Pever irregular. Referes viriable and unequal. Temperature rarely above 102°, Pulse relatively slow. Cases with blood infection often show early purpuric rash.	Slight or no backache or von- ling. Back of hands and forearms free from eruption. Bruption chiefly on trunk. Successive crops on same part of body. Soft papules part of body. Soft papules hy 2 d day becoming unicel- lular vesicles within few hours.	Onset more insidious than for exactatian, cibicular torisilii- tis or streptococcal angina. Peve less (10° to 10° F). Pulse tends to show arriby. In thin A. The min uria. Membrane pultaceous, tends to spread and leaves bleed- to spread and leaves bleed- leaves bleed- to spread and leaves bleed- leaves bleed-
pased on neport of Committee of A. 1. 11. Association	Laboratory diagnosis	Cultures from nose and pharynx of carrier or spinal fluid of case. Sp. fluid shows increased pressure and turbidity from pus cells, Meningcocci may be found in pus cells or extracellular. Lenkocytsis. Blood cultures often positive in early cases.	Inoculation (Lymph) rabbit's cornea nega- tive for Guarnieri bodies as against vari- ola. Paul's test negative.	Cultures throat, nose or other affected surfaces on Lóffler's blood serum or egg media. C. diphtheriae shows polar granules and tradtaneous nijection of ½6, M. L. D. toxin shows inflamed area if immunity not present. (Schick test).
a on report of Co	Period of communicability	As long as meningeoccus recovered from posterion naso-pharynx. Persistent "car-ires (3 months or longer) more comenon than formerly supposed.	Until primary scabs have dis- appeared from skin and mucosal lesions.	Until bacilli dis- appear from dis- charges or lesions. Healthy carriers in for months. Persistent car- ires offen show virulent bacilli in tonsillar crypts.
Dasc	Mode of transmission	Contact, drop- let infection let message or coughing of carriers or cases. Well admired danger ous to children tharry danger ous to children tact by articles freshy soiled by shifected persons.	Contact with patient or articles soiled by discharges of lesions.	Contact with discharges from sease or carrier. For mites or through milk or milk products.
	Incuba- tion period	2 to 10 days (common-ly $\eta$ ).	14 to 21 days.	2 to 5 days. Longer when car- rier stage precedes case.
	Cause and source	i. Carbrospinal Mentagitis. Messeria in- tracelularis macopharyn- gaal and mouth discharges of person with dis- ease and car- ners.	a. Chicken Pox (Varicella). Probably filtrable virus. Skin and mucous membrane lesions.	3. Diphtheria. Calphtheria. Throat, nose, conjunctiva, vagna.

servation to be promptly immunized by antitoxin. Prophylactic injection of anti-diphtheria serum protects only for 10 days. Immunization with toxoid more lasting and valuable. Disinfect fomites. Pasteurize milk.	Isolate patient 8 to 10 days. Keep contacts from school 3 weeks. No quarantine Eruption most marked about mouth and nose. Extremities by crops in 24 hours. Throat conditional seek glands may simulate scarlatina. Drug and food cruptions may simulate.	Prompt recognition of early symptoms and isolation during acute stage.  No proven method of immunization. During epidemics avoid crowds, well recognized climeal trointestinal, bronchial, nervous and gastrointestinal probably. Secondary infections in your reducing different parts of body, the vivia reducing resistance. Complicating preumonias and mid-ling pneumonias and mid-ling pneumonias and mid-ling pneumonias and mid-ling neuronias and mid-nervous and physical nervous and physical min.
geal palsies may come on (post-diphtheritie).	Rose-red macular throat eruption. Rose-pink skin eruption first appears on face. Disappears in 1 or 3 days. Course mid. Presence hybrid rash—morbilliform on face, scarlatinal insurface thigh, characteristic. Coryza generally slight.	Abrupt onset. Marked pros- tration. Muscular sore- ness, especially ocular and less boujunctival injection. Pulse slow relative to fever. Pulse slow relative to fever. Which show rapid rise to 102*-104. Epistaxis and protracted cough common.
	None. Plasma cells may be numerous.	In absence of secondary infections, a leukopenia.
	Dight days from onset of disease.	Undetermined. Probably during first; 7 days or even before symptoms.
	Direct contact with patient or fresh oral secretions.	Direct and indi- rect contract. Conjunctival atrium by drop- let. infectival possible. Fresh- ity soiled fo- mities have been incriminated.
	days.	Short. usu- ally 24 to 72 hours.
	4. German Meusias (Rötheln or rubella). Cause unknown, possi-tunknown, possi-tun a para a para a para by a virus. Ord and possibly nasal secritions.	5. Influenco. Pilitable virus. Influencoman secondary invader. Nasal secretions

## COMMON COMMUNICABLE DISEASES (Continued)

Cause and source	Incuba- tion period	Mode of transmission	Period of communicability	Laboratory diagnosis	Salient clinical features	Methods of control and remarks
6. Measles (Morbilli). Fil- trable virus. Oral and nasal secretions.	7 to 14 days (usually 10).	Direct contact with patient or through in- direct contact by means of articles freshly soiled with oral tons.	While oral and nasal mucous membranes involved. 5 days before and 5 days after rash.	Leukopenia with de- crease of lymphocytes before appearance of Koplik spots.	First 3 days coryza with marked fever and malaise. Kopilic spots on 1st or 2d day. Characteristic rash about 4th day, first on face. Crescent grouping. Face appears swollen. Conjunctivitis and bronchiis common. Secondary bronchoperators.	Isolate patient during period of communicability. Quarantine children exposed to infection from school and public gatheringst ddays. Isolate contacts showing 1°P. rise in temperature. Arthrificial immunication with convalescent serum.
7. Mumps. Epidemic parotitis. Secretions of mouth and possibly nose. Filtrable virus.	days. Usu-ally 18 days. Common maximal is 21 days.	Direct contact with case or articles freshy soiled with scretions cretions mouth or, possibly, nose.	Unknown but probably until parotid gland re- turns to normal.	Relative increase in mononuclears of blood.	Pain on opening mouth, chewing or swallowing. Parotid enhangement front and below ear of one or both sides. At times other salivary glands involved. Stomatits. Orchits and rarely energhalitis.	Isolate patient for period of presumed infectivity. Outantine exposed chill the from school or public gatherings at least 14 days. No immunization. In early diagnosis look for infammation of Steno's duct.
8. Poliomyelitis. Filtrable virus. Nose and throat discharges.	3 to 10 days (common- ly 6 days).	Direct contact with case or carrier. Indirectly by articles freshly soiled with virus.	Not known. Apparently not more than 21 days from onset of disease.	Usually slight leukopenia. Occasionally moderate leukocytomics. Sp. fluid clear, moderate lymphocytosis, increased protein and pressure.	Onset may resemble common of the behie diseases of childhood or meningitis. Fully developed flaced paralysis, most often lower extremities (75,8) comes on from 1 to 7 days.	Isolation of cases. Quarantine of exposed children also of adults who deal with children for the days from exposure to case. Healthy carriers supposed to be common.
9. Scarlet Fever (Stariatina). Streptococcus Scarletina e. Discharges nose, throat and ear, and supurating glands.	2 to 7 days (usually 3 or 4 days).	Direct contact with case or articles soiled by interfers soiled discharges or by contaninated milk.	Three weeks from onset of disease and until all abnormal discharges have casased and open sores healed.	Nasopharyngeal cultures stroptococci. Marked ed leukovytosi ns ever cases. May be some eosinophilia. Examine urine for albumin. Schultz-Charlton reaction. Dick test.	Sudden onset with vomiting, tendency to high fever tachlycardia, anguna and hellaged glands at angle of jaw. Bolied-lobster-like eruption by end of 24 hours appearing first on neck and upper chest. Most marked bott armiglist and region of Scarpa's triangle. Consider possibility of drug or serum rashes. Lok for strawberry tongue. Anguna strawberry tongue. Anguna strawberry tongue. Anguna strawberry tongue. Anguna	Isolate case 38 days. Carry out Dick test on contacts and nurses. Susceptibles not show- ing heenolytic strepto- coci in throat or if showing streptococci but no fever or other symp- toms to be actively im- munized with scarlet fever foxin. Suscepti- bles with haemolytic streptococci in throat,

COMMON COMMUNICABLE DISEASES (Continued)

	Methods of control and remarks	fever or other symptoms, for receive scarlet flover sa antitotin (simfleant to neutralize 90,000 S.T. Djsimfect all discharges. Paeteurize milk.	de General vaccination in- fancy. Revaccination is school entry or in times of unistal preva- tion is creened wards. In Disinfection of all dis- fluctuation of all con- tacts to days or until representation. In protected ty vaccina- tacts fo days or until protected ty vaccina- tacts for the con- t	Isolate chiid 2 weeks after "who op." Dismitect discharges, Vaccina- tion possibly of prophy- lactic value.
	Salient clinical features	may be membranous and show streptococci in smears. Deguamation in flakes. Nephritis a common sequel.	High fever, marked head- each, backache, and vomit- ing for 3 days. Rarely ini- tial scarlatinal or measly rashes on 4d day. Shotty papular rash at hat line and wrists on 4dt day with fall of fever. Bruption on trunk and extremities follows facial one in 24 hours. Multilocu- lar vesicles on 6th day. Um- lar vesicles on 6th day. Um- lar vesicles on 6th day. Same of fever on 8th day. Same type lesion on same region	Onset with coryza or bron- chitis for 3 to 14 days before development of characteris- tic "whoop." Secondary bronchopneumonia serious.
,	Laboratory diagnosis		Reduction of polynuclears of ymphocytes until pustule formation—then leukocytosis. Iron maxed (pyogent) in fection. Immediate vaccination reaction (34 to 48 hours) shows immunity to smalpox. Inoculation tests (Paul's, etc.)	Primary lenkocytosis, then lymphocytosis, then cosmophilia. Ex- amine smear from spu- tum of early stages and culture same.
	Period of communicability		From first symptoms to disappearance of scabs and crusts.	Particularly con- tagious in early stages before thar- acteristic whoop. Communicability probably lasts 2 w e e ks a f t e r whoop.
	Mode of transmission		8 to 18 days. Personal consommers tact, also by up to 21 articles soiled days. charges, Plies may transfer virus.	Direct contact with cases or articles freshly soiled by dis- charges.
	Incuba- tion period		8 to 18 days. Sometimes up to 21 days.	Within 14 days.
	Cause and source		10. Smellpox Variola). Cause a filtrable virus. Lesions of skin and mucous membranes.	II. Whooping cough (Pertussis). Bordet-Gengou bacillar. Bronchial or larry yngeal secretions.

## M. LABORATORY PROCEDURES USEFUL IN DIAGNOSIS, INDEXED BY DISEASES

This index has been compiled to assist in the selection of laboratory procedures which are likely to be of value in the diagnosis of the diseases listed, particularly those which can not be recognized by their clinical features alone. Only those of major importance can be given. The statements in the index apply to the average case, and many are subject to limitations or qualifications for which the text should be consulted. To be of value, laboratory tests must be performed with such precision that the probable technical error is well within the limits of the physiological variation. The results can be interpreted correctly only in conjunction with all the information available concerning the patient. This responsibility belongs to the clinician, and should not be expected of, or entrusted to a laboratory technician.

Abscess.—Aspirate contents with sterile pipette or syringe and culture on agar and blood agar plates. Stain films by Gram and, if indicated, for acid-fast bacilli. In special cases inoculate animals. Usually a neutrophilic leukocytosis. Sedimentation rate increased. Urine may contain proteose.

Abscess of Liver, Amoebic.—Aspirate contents aseptically and culture on blood agar plates (no growth unless secondarily infected). Pus resembles anchovy sauce. Stained films usually show detritus with few pus cells. Amoebae found in fresh preparations only after drainage has been established. Examine faeces for trophozoites and cysts. Moderate neutrophilic leukocytosis if acute. Monocytosis. Roentgenogram.

Abscess, Lung.—Sputum abundant, purulent, often foul; may layer on standing. Culture aerobically and anaerobically. Stain by Gram and Ziehl-Neelsen methods and examine fresh material for spirochaetes and fusiform bacilli, preferably with dark field. Look for elastic fibres. Exploratory aspiration in some cases. Leukocytosis. Roentgenogram. Differentiate from tuberculosis, fungus infections, ruptured liver abscess, tumor.

Abscess, Tuberculous.—If stained films of the pus are negative for pyogenic organisms, stain for acid-fast bacilli. Concentrate with alkali if necessary. Make cultures and inoculate a guinea pig. Leukocyte count variable. Sedimentation rate increased.

Achylia Gastrica.—Do fractional gastric analysis after histamin injection. (See p. 752.)

Acidosis.—If possible test CO<sub>2</sub> combining power of plasma. Alternatives: Test CO<sub>2</sub> tension of alveolar air. Measure titratable acidity + ammonia output in urinc. Determine tolerance for NaHCO<sub>3</sub>. Look for ketone bodies in urine. Test pH of urine. Ammonia in urine increased at expense of urea. (See p. 687 and 730.)

Actinomycosis.—Look for yellow "sulphur" granules in pus or sputum. Press out granules between slides and stain by Gram's method. The central mycelium is Gram-positive while peripheral "clubs" are Gram-negative. Culture anaerobically. (See p. 230.)

Acute Yellow Atrophy of Liver.—(See Liver, necrosis of.)

Addison's Disease.—In crises, marked reduction in serum sodium and total base, and in chlorides and bicarbonate. High blood non-protein nitrogen and urea. Hypo-

### USEFUL LABORATORY PROCEDURES

glycacmia. "Flat" glucose tolerance curve. During remissions salt restriction may precipitate a crisis (dangerous). Hypersensitive to insulin injections. Roentgenogram for calcified areas in adrenals.

Agranulocytosis.—See Malignant neutropenia.

Alkalosis.—Test CO<sub>2</sub> combining power of plasma. Test pH and titratable acidity of urine. Look for signs of tetany.

Allergy.—See Hypersensitiveness.

Amoebiasis.—Note gross and microscopic appearance of the stools. (See p. 413.) Examine facces for trophozoites and cysts. May show monocytosis. Differentiate from bacillary dysentery and chronic (non-specific) ulcerative colitis.

Amyloidosis.—For changes in blood and urine see "Nephrosis." Make Congo red

test (p. 719). Search for evidences of chronic suppuration or tuberculosis.

Anaemia, Aplastic.—(See p. 358.) Qualitative changes in red cells slight. No signs of regeneration. Marked neutrophilic leukopenia and thrombocytopenia with purpura. Sternal marrow shows few erythroblasts or myelocytes.

Anaemia, Haemolytic.—(See p. 361.) Positive indirect van den Bergh. High icterus index. Urobilin increased in urine and faeces. Reticulocytes usually increased. Leukocytosis. In fulminant cases look for haemoglobinuria.

Anaemia, Hypochromic.—(See p. 347.) Cells very pale, usually small. Color index and saturation index low. Corpuscular volume usually low. Icterus index normal or low.

Anaemia, Idiopathic Hypochromic.—Largely limited to women 20 to 50 years old. Usual features of hypochromic anaemias marked. Leukopenia frequent. Subacidity or achlorhydria, usually even after histamin. Reticulocyte crisis follows administration of large doses of iron. Differentiate from chronic post-haemorrhagic anaemia, chlorosis, cancer, hookworm anaemia. (See p. 348.)

Anaemia, Pernicious.—(See p. 353.) High volume index and high color index. Marked anisocytosis, macrocytosis, poikilocytosis. Megaloblasts in severe untreated cases. Evidences of haemolysis during progressive stages. Leukopenia with hypersegmented neutrophiles. Blood cholesterol reduced. Achlorhydria after histamin. Reticulocyte crisis follows administration of potent liver extract in severe cases.

Anaemia, Posthaemorrhagic, Acute.—Features of hypochromic anaemia present in moderate degree. Usually reticulocytosis, leukocytosis and increase in platelets. Normoblasts may appear after third day.

Anaemia, Posthaemorrhagic, Chronic.—Features of hypochromic anaemia marked. Low color and saturation index. Evidences of regeneration variable. In late stage may show aplastic type of anaemia. (See p. 347.)

Anaemia, Sickle Cell.—Seal fresh moist preparations and observe for 1 to 24 hours for sickled red cells. (Rare in stained films.) Severe cases show many normoblasts, reticulocytosis, leukocytosis, increased platelets. Look for macrophages containing red cells. Roentgenograms of skull may show changes. Limited to negroes.

Anaphylaxis.—See Hypersensitiveness.

Ancylostomiasis.—Examine faeces by concentration methods for characteristic ova. (See p. 500.) Examine blood for anaemia (hypochromic, microcytic) and eosinophilia.

Angina, Streptococcal.—Make cultures on Löffler's serum and blood agar plates. Stain films with Löffler's methylene blue and Gram. Differentiate from diphtheria and Vincent's angina.

Angina, Vincent's.—See Fuso-spirochaetosis.

Anthrax.—(1) Malignant pustule. Examine material from pustule directly in stained films and hanging drop for large, Gram-positive, non-motile bacilli. Culture on agar. Inoculate a mouse or guinea pig subcutaneously. Blood cultures rarely positive. (2) Woolsorters' disease. Examine sputum in a similar way.

Appendicitis.—Neutrophilic leukocytosis with increase in juvenile forms. Differentiate from renal and pelvic infections.

Arsenic Poisoning.—Examine urine for arsenic (in the late stages, the hair). Examine urine for albumin, casts, renal epithelium, urobilin, bile. Examine blood for non-protein nitrogen or urea, creatinin, bilirubin. Test renal function and liver function. Test for acidosis.

Arthritis, Acute.—Neutrophilic leukocytosis. Make blood culture. In special cases aspirate fluid, culture on blood agar, and stain films by Gram.

Arthritis, Chronic Infectious.—Joint fluid usually sterile. (See p. 644.) Agglutination tests or intracutaneous allergic tests with strains of streptococci. (See p. 34.) Streptococci can sometimes be cultivated from excised lymph nodes draining affected joints. Use deep tubes of serum glucose agar. Sedimentation rate increased. Occasional leukocytosis. Blood uric acid normal.

Ascaris Infection.—Examine faeces for characteristic ova. Worms occasionally found in faeces or vomitus.

Ascites.—Culture fluid on blood agar. Determine whether transudate or exudate. (See p. 643.) If tuberculosis is suspected digest sediment with alkali, and examine centrifugate by staining for acid-fast bacilli, special cultures and guinea pig inoculation. See also cirrhosis of the liver, oedema, nephritis.

Asthma.—Examine sputum for eosinophiles, Charcot-Leyden crystals, Curshmann's spirals. Make culture from sputum for possible causative organism in cases due to respiratory infection. Eosinophilia. Make cutaneous allergic tests. Severe cases with cyanosis show decreased O-saturation in arterial blood. May show acidosis (CO<sub>2</sub> excess) with high plasma bicarbonate.

Bacteriaemia.—Make blood culture. Usually neutrophilic leukocytosis with shift to the left.

Bacteriuria.—See Urinary tract infections.

Balantidium Infection.—Look for large motile ciliates in faeces.

Banti's Disease.—Moderate to severe anaemia, usually hypochromic in type. Leukopenia. Test fragility of the red cells to exclude haemolytic jaundice. Make van den Bergh test and determine icterus index. Test liver function. Examine faeces for occult blood.

Blackwater Fever.—Haemoglobinuria: pink foam to urine; test filtrate for haemoglobin spectroscopically and by benzidine or orthotolidine test. Malarial parasites found in thick blood films in some cases. Leukopenia. Monocytosis. Examine blood serum for haemoglobin and bilirubin (van den Bergh test). Donath-Landsteiner test negative.

Blastomycosis.—Examine sputum, pus, or scrapings from margins of ulcers in 10% KOH for spherical, budding yeast cells with highly refractile, double-contoured walls. Make culture on glucose agar plates.

Botulism.—Inject an infusion of the suspected food into a guinea pig. (See p. 65.) Culture anaerobically on glucose agar. Culture may be kept in a dark place at room temperature.

Bronchiectasis.—Sputum abundant, purulent, often foul, tends to separate into three layers on standing. Small haemoptyses common.

Bronchitis.—Culture sputum on blood agar plates. Stain film by Gram. In special cases inoculate a mouse.

Brucellosis.—Make blood culture at onset of febrile paroxysm; incubate in atmosphere of 10% CO<sub>2</sub>. Make cultures from urine, faeces, and local foci in special cases. Guinea pig inoculation sometimes successful. After fifth day make agglutination tests; titer of 1-100 or over is diagnostic. Intradermal test with vaccine. Relative lymphocytosis, often leukopenia. Differentiate from typhoid fever, tuberculosis, malaria, kala azar.

Carbon Monoxide Poisoning.—Test blood for CO spectroscopically and by Sayers-Yant method. Secure blood at earliest possible moment and protect from air.

Cerebrospinal Fever.—See Meningitis, meningococcus.

Cestode Infections.—Examine faeces for ova, which are not always present. If a segment is obtained, press between two glass slides and examine the branchings of the uterus.

Chancroid—Ducrey's Bacillus.—Examine smears for short, Gram-negative coccobacilli occurring in chains. Culture material aspirated from bubo in sterile clotted human or rabbit blood which has been inactivated at 56°C. for 30 minutes. Syringe and media must be warm.

Chlorosis.—Hypochromic microcytic anaemia, sometimes severe, in adolescent girls. Low saturation index. Gastric acidity normal. Differentiate from chronic posthaemorrhagic anaemia.

Cholecystitis.—May be moderate leukocytosis. Fluid obtained by duodenal drainage may show bile-stained pus cells and other abnormalities. (See p. 761.) Culture on blood agar plates. Van den Bergh test.

Cholelithiasis.—Examine blood and urine for bilirubin (van den Bergh test). Bile obtained by duodenal drainage often shows increase in cholesterin crystals and calcium bilirubinate precipitate, and evidences of cholecystitis (q.v.). Blood cholesterol increased with obstructive jaundice. Roentgenogram (Graham test).

Cholera.—Smears from flecks in rice-water stools show many vibrios with "fish in stream" arrangement. Culture on Dieudonné plates. If sparse, use enrichment method. Identify organism with cholera immune serum. After fourth day test serum for agglutinins. Intense dehydration with high blood counts, high plasma proteins and high specific gravity of blood. Anuria with high non-protein blood nitrogen. Acidosis from loss of base. Depletion of chlorides. Differentiate from food poisonings, arsenic or antimony poisoning, bacillary dysentery, algid pernicious malaria.

Chyluria.—Centrifuge urine and examine for filarial larvae. Examine blood at night for filarial larvae (not always present). Urine contains many highly refractile fat globules soluble in ether.

Cirrhosis of Liver.—Do Wassermann or Kahn test. Icterus index and van den Bergh test. Test urine for bilirubin and urobilin. Make tests of liver function and Takata-Ara test (p. 769) or determine A/G ratio. Examine faces for occult blood. Late cases may show macrocytic anaemia. (See also Ascites, Banti's disease, Liver, necrosis of.)

Coccidioidal Granuloma.—Examine pus or scrapings from ulcers in 10% KOH for large yeast-like cells which may contain endospores. Biopsy if necessary. Culture

on glucose agar. Make blood culture. Exclude tuberculosis by repeated stains, cultures and guinea pig inoculation. (See p. 214.)

Colitis, Chronic Ulcerative.—Examine faeces or preferably scrapings from ulcers (proctoscope) for pus, blood, mucus. Exclude amoebic and bacillary dysentery by fresh warm-stage preparations, cultures and agglutination tests. Neutrophilic leukocytosis, often secondary anaemia, increased sedimentation rate. Roentgenograms.

Colitis, Mucous ("Spastic Colitis").—Examine faeces for mucus in large masses, containing epithelial cells, often eosinophiles, no pus cells, no blood. No leukocytosis, normal sedimentation rate. Roentgenograms.

Coma.—Examine urine for sugar, ketone bodies, albumin, casts, blood. Examine blood for sugar, CO<sub>2</sub> combining power, non-protein nitrogen or urea, and in special cases for alcohol and CO. Consider possibility of other poisons. Examine spinal fluid (caution, see p. 63r), especially for pressure, presence of red cells and xanthochromia (subarachnoid haemorrhage). Make leukocyte count and blood culture if febrile.

Conjunctivitis.—Stain smear by Gram's method and with dilute carbol fuchsin. Culture secretion on blood agar and plain agar. (See p. 603.)

 $\begin{tabular}{lll} \textbf{Coronary} & \textbf{Thrombosis.} & \textbf{-} \textbf{Neutrophilic} & \textbf{leukocytosis.} & \textbf{Accelerated} & \textbf{sedimentation} \\ \textbf{rate.} & \end{tabular}$ 

Cyst.—Examine fluid for echinococcus hooklets and scolices. Test for pancreatic ferments and for urea.

Cystitis. - See Urinary tract infections.

**Dehydration.**—Examine blood for increase in haemoglobin concentration and in plasma protein (the best gauge). Specific gravity is increased.

Dengue.—Neutrophilic leukopenia.

**Dermatomycoses.**—Examine scrapings from skin in 10% KOH for fungi. Culture on Sabouraud agar.

**Diabetes Insipidus.**—No albuminuria or glycosuria. No nitrogen retention. Ability to concentrate urine lost, restored temporarily by pituitrin injections.

**Diabetes Mellitus.**—Examine urine for sugar and ketone bodies. Examine blood (fasting) for sugar, cholesterol. In doubtful cases do glucose tolerance test. Make tests for acidosis (q.v.). In severe cases test for decrease in blood bases (or chlorides) and for dehydration.

Diphtheria.—Make smears and cultures on Löffler's serum or whole egg medium. Stain by Gram's and Löffler's or Neisser's method. Look for parallel rods containing polar granules. In special cases isolate on tellurite blood agar and inject guinea pig with broth culture filtrate as test for virulence. Make Schick test on contacts. Differentiate from streptococcus and Vincent's infections.

**Diphyllobothrium Infection.**—Operculated ova in faeces. If segments are obtained, press one between two glass slides and look for characteristic rosette-shaped uterus. Macrocytic anaemia occurs in very rare instances.

Dracunculus Infection.—Moisten blister or ulcer with a few cubic centimeters of water. Examine fluid excreted by worm for striated larvae.

**Duodenal Ulcer.**—Make gastric analysis (for hyperacidity, blood, hypermotility). Examine faeces for blood. Roentgenograms.

Dysentery, Amoebic.—Examine mucus from fresh warm stool (warm stage) for amoebae actively putting forth blade-like pseudopodia. If necessary pass rectal tube, give saline purge, or scrape base of ulcer through a proctoscope. Pathogenic amoebae often contain red cells. Examine faeces for four-nucleated cysts. Smear of faeces

shows granular detritus, often Charcot-Leyden crystals, no pus cells. Monocytosis. No eosinophilia.

Dysentery, Bacillary.—The sanguinolent mucus contains many pus cells and many phagocytic endothelial cells. Emulsify mucus in sterile broth or salt solution and plate on Teague, Endo or desoxycholate medium. Identify organisms isolated by agglutination with immune sera. Neutrophilic leukocytosis. After 7 to 10 days make agglutination tests.

Echinococcus Disease.—Examine fluid from cyst for hooklets. Eosinophilia. Make complement fixation, precipitin, or cutaneous tests with special antigen. (See p. 484.) Roentgenograms.

Elephantiasis.-See Filariasis.

Empyema.—Neutrophilic leukocytosis. Make Gram's stain of smear from pus. Culture on blood agar plates. Roentgenogram.

Encephalitis, Epidemic.—Examine spinal fluid. (See p. 630.) Make cultures and stains of sediment to exclude ordinary types of meningitis.

Endocarditis, Subacute Bacterial.—Make blood culture (repeatedly if negative). (Plates usually show *S. viridans*, rarely pneumococcus, gonococcus or Pfeiffer bacillus.) Moderate neutrophilic leukocytosis (inconstant), often increase in macrophages. Progressive anaemia. Increased sedimentation rate. Examine urine for albumin, casts, blood (renal emboli).

Enteritis, Tuberculous.—Stain smear from faeces for acid-fast bacilli. There are often present in faeces acid-fast, spore-like bodies which should not be mistaken for tubercle bacilli. Tubercle bacilli may be present in faeces when there is no involvement of intestine (swallowed sputum, particularly in children).

Eosinophilia.—Look for ova of intestinal parasites in faeces. Look for evidence of trichinosis, hydatid disease, filariasis, various skin diseases, asthma and other allergic reactions, and lesions of the bone marrow.

Erythremia. See Polycythaemia.

Exophthalmic Goiter.—High basal metabolic rate. Low blood cholesterol, high sugar. Glucose tolerance curve shows high peak, often prolonged. Glycosuria common. Often lymphocytosis.

Favus.—Place hair or portion of favus cup on a slide and examine in 10% KOH for mycelium and spores which are very irregular.

Filariasis.—Examine blood (day and night) for larvae, either in fresh moist preparation or better in thick films stained by Giemsa. (In elephantiasis larvae are often absent from blood.) Aspirate lymph varix or hydrocele and examine sediment for larvae. Examine sediment of chylous urine or ascitic fluid. Often cosinophilia. Complement fixation test positive with special antigen.

Flagellates, Intestinal.—Examine faeces emulsified in salt solution for motile flagellates. Emulsify another portion in Gram's solution to study flagella. Stain smear with Giemsa's stain for encysted forms, which may be confused with Blastocystis. (See p. 436 and 870.)

Furunculosis.—Determine blood sugar, or preferably make glucose tolerance test. Make cutaneous test with staphylococcus toxin. (See Abscess.)

Fusospirochaetosis (Vincent's Angina and Stomatitis).—Stain films of material from the depths of the lesion with dilute carbol fuchsin or Fontana's stain. (The spirochaetes and fusiform bacilli are present in large numbers; a few may be present in normal mouths.) Make cultures on Löffler's serum and blood agar to exclude diphtheria and streptococcal infection. Examine blood to exclude leukaemia and

agranulocytic angina. There may be a marked lymphocytosis. Exclude syphilis by Wassermann or Kahn test. In pulmonary infections examine *perfectly fresh* sputum in stained films and dark-field preparations. See Abscess of lung.

Gas Gangrene.—(Infections are usually mixed; important organisms are Cl. welchii, Cl. oedemaliens and Cl. oedemalis-maligni. Precise identification difficult but important because of serum treatment.) Make hanging drop and stain smears from exudate by Gram and for capsules. (All are Gram-positive; Cl. welchii is encapsulated and non-motile; the other two are motile and non-encapsulated.) Make anaerobic cultures in litmus milk and glucose agar tubes. (Cl. welchii causes gas formation and disruption of the tube over night.) Blood cultures (anaerobic) may be positive. (For isolation and identification by inoculation into immunized animals see p. 63.)

Gastric Cancer.—Examine fasting stomach contents for retention of food, lactic acid, 'Boas-Oppler bacilli, occult blood. Test meal shows achlorhydria and delayed motility. Occult blood in faeces. Usually anaemia, hypochromic microcytic; often neutrophilic leukocytosis. Increased sedimentation rate. Roentgenogram.

Gastric Ulcer.—Examine fasting stomach contents for free HCl, food retention, sarcinae, blood. Test meal shows normal or high acid, often delayed emptying. Occult blood in faeces, often intermittent. Roentgenogram.

General Paresis.—Examine spinal fluid: shows increased cell count, positive globulin test, paretic colloidal gold curve, positive Wassermann and Kahn tests. Blood shows positive Wassermann and Kahn tests.

Glanders.—Smears from pus show characteristic Gram-negative bacilli, parallel beaded rods. Culture on acid glycerin agar and potato. Inoculate male guinea pig intraperitoneally. (See p. 92.) Complement fixation and agglutination tests may be positive. (Mallein tests in animals.)

Glycosuria.—Examine urine for sugar and apply special tests for glucosc. Fasting blood sugar and glucose tolerance test. Consider renal diabetes and other causes for glycosuria. (See pp. 661 and 666.)

Gonococcus Infection.—Gram's stain of smear from urethra, cervical canal or eye shows intracellular, Gram-negative diplococci. Make culture. (See p. 847.) Complement fixation test useful in chronic infections.

Gout.—Uric acid in blood often increased. Examine crystals curetted from tophus, if present, by murexid test. Uric acid excretion in urine increased at onset of acute attack.

Granuloma Venereum.—Stain scrapings from ulcers for Donovan bodies in large mononuclear cells (Wright or Giemsa).

**Haematuria.**—Examine sediment for red cells. Apply orthotolidine test. (For causes see p. 700.)

Haematuria, Egyptian.—(See Schistosomiasis.)

Haemochromatosis.—Examine urine for sugar and for haemosiderin granules in sediment. Fasting blood sugar or glucose tolerance test if in doubt. Make van den Bergh test. Test liver function. Examine piece of excised skin for haemosiderin.

Haemoglobinuria.—Examine centrifuged sediment for intact red cells. "Shadow cells" may be found with much debris. Test filtered urine for haemoglobin spectroscopically and by benzidine or orthotolidine test.

Haemophilia.—Prolonged coagulation and "prothrombin" time. Normal bleeding time, normal platelet count, normal clot retraction. Negative tourniquet test. May show posthaemorrhagic anaemia. (A congenital abnormality limited to males.)

Hodgkin's Disease.—Examine section of excised lymph gland. The blood may show moderate leukocytosis, monocytosis (10% to 15%), increased platelet count, occasionally eosinophilia (no constant changes). In late stages, a severe anaemia. May try Gordon's test. (See p. 377, reliability not yet established.)

Hookworm Infection.—(See Ancylostomiasis.)

Hypersensitiveness.—Test for hypersensitiveness to pollens, animal hair, foods, etc., by cutaneous tests or intracutaneous injections of suitable extracts, or in special cases by patch tests. To determine if a patient is sensitized to a serum, inject intradermally 0.1 cc. of a 1-10 dilution of the serum. If he is sensitized, an urticarial wheal will develop within 10 or 15 minutes. A drop of serum in 1-10 dilution may be instilled into the conjunctiva (see p. 253). Sensitization to quinine may be shown in occasional cases by the production of a wheal shortly after 10% solution of a quinine salt is applied to a scratch.

Hypertension, Essential.—Examine urine and test renal function, especially by concentration tests, to determine extent to which kidney is involved.

Infectious Mononucleosis.—Leukocytosis with high lymphocytosis (up to 90%). Many pathological lymphocytes. (May not appear until several days after onset and may persist for months.) After 5 to 10 days test serum for "heterophile" agglutinins for sheep red cells.

Intestinal Parasites.—Examine faeces for ova.

Jaundice, Catarrhal (Hepatitis).—Test urine for bile. High icterus index. Positive van den Bergh reaction; type variable. (See p. 765.) Faeces show diminished or absent bile pigments, increased fat. Test liver function. Differentiate from infectious jaundice, arsenic and phosphorus poisoning, types of obstructive jaundice.

Jaundice, Familial Haemolytic.—Usual features of haemolytic anaemia marked. Fragility of red cells in hypotonic salt solution increased. Reticulocytes much increased. Red cells have normal volume but diameter is small (more globular than normal). Look for evidences of cholelithiasis.

Jaundice, Infectious (Weil's Disease).—Examine blood (first 3 days) for leptospira in stained films or dark-field preparations (sparse). Preferably inoculate a guinea pig with 1 to 5 cc. of blood intraperitoneally and at autopsy examine the liver for leptospira. Blood cultures may be made in Fletcher's medium and incubated at 25° to 30°C. After the twelfth day examine urine sediment for leptospira by smear and guinea pig inoculation. If stock cultures are available, after 10 to 14 days patient's serum may give a positive adhesion phenomenon or agglutination or protection test. Leukocytosis.

Jaundice, Obstructive.—Blood shows high interus index and positive direct van den Bergh tests, increase in cholesterol and often in diastase. Coagulation time increased. Urine shows bilirubin, no urobilin. Faeces show increased fat and (if obstruction is complete) no bilirubin or urobilin. Fragility of red cells normal or diminished.

Kala Azar.—Culture blood on N.N.N. medium. Search for Leishmania within the leukocytes and monocytes in blood films, either directly or from the buffy coat in which the leukocytes are concentrated, after centrifugalization of citrated blood. If not found, examine material obtained by splenic puncture or more safely by puncture of the liver or sternal bone marrow. (They are found occasionally in excised lymph glands or aspirated gland juice.) Blood globulin markedly increased. Make formolgel and antimony tests. Marked leukopenia with relative monocytosis. Differentiate from brucellosis, typhoid and paratyphoid fever, Banti's disease, chronic malaria, leukaemia.

**Ketosis.**—(1) Examine urine for ketone bodies. Test pH and titratable acidity of urine. Test for acidosis (q.v.). (2) When ketosis is deliberately induced, control the degree of ketosis by daily estimations of pH and ketone bodies in urine. (See p. 724.)

Lead Poisoning.—If exposure is recent, examine urine for lead. Blood shows early increase in reticulocytes and many stippled cells; later anaemia, in acute cases severe and haemolytic in type. Fragility of red cells decreased. Often neutrophilic leukocytosis. Examine urine for albumin and casts. In late cases test renal function.

Leishmaniasis.—See Kala azar and Oriental sore.

Influenza.—Leukopenia and granulocytopenia. Frequent secondary infections, particularly of the respiratory tract.

Leprosy.—Diagnosis depends upon demonstrating leprosy bacilli in smears stained by Ziehl-Neelsen method. Decolorize lightly with 20% aqueous  $\rm H_2SO_4$ . Morphology characteristic. Usually abundant in material from granulomata or scrapings from ulcers, especially from nose. If no lesions are evident, examine scrapings from nasal mucous membrane or skin clips from ear lobe. Examine blood during febrile periods; make thick films, dehaemoglobinize and stain as above, or stain films from sediment after digestion with alkali. Cultures and animal inoculation useless except to exclude tuberculosis. Wassermann reaction positive in about 60% of the cases.

Leukaemia, Acute.—Total leukocyte count ranges from normal to 150,000, occasionally much reduced. Diagnosis depends upon presence of many primitive leukocytes with non-granular basophilic cytoplasm and nuclei showing fine chromatin network and nucleoli. Progressive anaemia with normoblasts. Reduced platlet count. Prolonged bleeding time. Increased basal metabolic rate.

Leukaemia, Chronic Lymphatic.—Leukocyte count usually from 20,000 to 100,000, with 75 to 99% small lymphocytes, a few lymphoblasts and Rieder cells. May be a few myelocytes. Progressive anaemia. Normoblasts rare. Basal metabolic rate increased. Blood uric acid high. Marrow (biopsy or sternal puncture) shows increase in lymphocytes.

Leukaemia, Monocytic.—Leukocyte count ranges from normal to 400,000 with 20% to 90% monocytes and monoblasts. Examine fresh supravitally stained films as well as fixed films. Myelocytes usually present, may be numerous. Usually marked anaemia and reduced platelets.

Leukaemia, Chronic Myelogenous.—Total leukocyte count usually from 200,000 to 500,000, with a large proportion of myelocytes. In terminal stage many myeloblasts. Progressive anaemia. Normoblasts nearly always present, often numerous. Platelets increased. Basal metabolic rate increased. Blood uric acid high.

Liver, Necrosis of.—Test liver function. (See p. 766.) Bilirubin in blood increased. Urobilinuria. In advanced stages shows: Increase in ratio of  $NH_3$ /urea in urine, with leucin and tyrosin crystals, usually only after concentration. High non-protein nitrogen with low urea in blood. Hypoglycaemia. Decreased fibrinogen. Prolonged coagulation time. Decreased sedimentation rate. Acidosis may occur.

Lymphogranuloma Inguinale.—Frei test.

Madura Foot.—Discharge contains fish-roe granules which show mycelium and peripheral club-like structures.

Malaria.—Examine thin films stained by Giemsa or Wright's stain. If negative, stain thick films. To identify an object as a malarial parasite in stained films, one should be able to make out at least two of three characters: (1) chromatin; (2) bluish or greenish cytoplasm; and (3) pigment. Crescents are diagnostic for malignant

tertian, equatorial banding for quartan. Marked irregularity of outline of parasite and the presence of Schüffner's (reddish) dots in cytoplasm of red cells suggests benign tertian. Leukopenia with monocytosis. Leukocytosis during the paroxysms. Anaemia of haemolytic type. Plasma globulin increased. About 15% show positive Wassermann reaction during febrile periods. May try provocative procedures. In special cases make sternal (or splenic) puncture.

Malignant Neutropenia.—Leukopenia, becoming extreme, with disappearance of granulocytes. Red cells, platelets, coagulation factors usually normal. Granulocytes in marrow greatly reduced. Make blood culture to exclude sepsis. Stain films for Vincent's organisms. Differentiate from sepsis, acute leukopenic leukaemia and aplastic anaemia.

Measles.—Blood shows neutrophilic leukopenia. Diazo-reaction in urine usually precedes cruption and is not found in German measles. (For use of convalescent serum, see p. 102.)

Melanosarcoma. —Urine usually turns black after exposure to the air because of the formation of melanin. (This occurs, rarely, in other conditions, e.g., alkaptonuria.) Does not reduce Benedict's solution.

Meningitis, Lymphocytic Chorio.—Spinal fluid shows increased pressure, slightly increased globulin. 50 to 2000 lymphocytes, meningitic colloidal gold curve, sterile cultures. Inoculate guinea pig subcutaneously, or mice intracerebrally. Virusneutralizing antibodies in blood after sixth week. Differentiate from encephalitis, poliomyelitis, tuberculous meningitis, neurosyphilis.

Meningitis, Meningococcus.—Blood cultures often positive in early cases and in simple meningococcaemia. Spinal fluid purulent, under high pressure. Culture immediately on warm blood agar. Examine stained film for Gram-negative intracellular or extracellular diplococci. Fluid gives a precipitin reaction with polyvalent antimeningococcus serum. If cloudy fluid is obtained, give serum immediately. Neutrophilic leukocytosis. Differentiate from septic meningitis, tuberculous meningitis, poliomyelitis, encephalitis, benign lymphocytic choriomeningitis. Detect carriers by cultures from posterior nasopharynx.

Meningitis, Tuberculous.—Spinal fluid clear or opalescent, shows increased pressure, increased globulin, lymphocytic pleocytosis, decreased sugar, markedly decreased chlorides. Stain for tubercle bacilli in fibrin web which forms on standing. Make cultures or inoculate guinea pig. Differentiate from benign lymphocytic choriomeningitis, encephalitis, poliomyelitis.

Mercury Poisoning.—Examine urine or gastric contents for mercury. Examine urine for volume (oliguria), albumin, casts, renal epithelium. Determine blood non-protein nitrogen or urea, and creatinin. If severe, test for acidosis. Renal function tests show impairment.

Myeloma, Multiple.—Bence-Jones protein usually found in urine. High plasma globulin (up to 9%), even without Bence-Jones proteinuria. May show high blood calcium and low phosphorus. Anaemia, occasionally erythroblastic, with leukocytosis and myelocytes. Roentgenogram.

Myxoedema.—Basal metabolic rate retarded (to -40%). Blood shows high cholesterol, low glucose. Low flat glucose tolerance curve. Anaemia, either macrocytic or hypochromic microcytic in type. Often lymphocytosis.

Nephritis, Acute and Chronic.—Examine urine, especially for albumin, casts, blood and pus cells. The presence of blood and red cell casts indicates an acute process.

In acute cases look for streptococcal throat infections. (See p. 34.) Test renal function. Determine blood non-protein nitrogen or urea, and if high, creatinin or phosphorus. If impaired, determine blood chloride or bases, and test for acidosis. Anaemia common in advanced stages. (See "Nephrosis.")

Nephrolithiasis.—Examine urine for blood and pus. The presence in *freshly* voided urine of clumped calcium oxalate or uric acid crystals is suggestive. Examine urine for cystin crystals. Make culture to determine whether pyelitis is present. Roentgenogram.

"Nephrosis"—"Nephrotic" Stage of Glomerular Nephritis.—Examine urine: Oliguria, high fixed specific gravity, marked albuminuria, many casts, epithelial cells with doubly refractile fat droplets, pus cells, but few or no red cells. Chloride excretion reduced. Phthalein excretion normal. Blood cholesterol very high, plasma albumin reduced, A/G ratio inverted. Chlorides may be high. No nitrogen retention. Congo red test positive. Sedimentation rate much accelerated. Basal metabolic rate retarded. Anaemia common. Look for evidence of intercurrent infections.

Ochronosis (Alkaptonuria).—Urine contains homogenetisic acid, which turns black on standing exposed to the air (especially if alkaline). Reduces Benedict's solution but not bismuth salts, and is not fermented by yeast. Rare anomaly of metabolism.

Oedema.—Examine urine for evidence of nephritis. Test renal function. Determine plasma proteins, A/G ratio, blood chlorides. See "Nephrosis."

Onchocerciasis.—Aspirate fluid from a nodule and look for motile larvae in fresh preparation, or stain by Giemsa. If negative, excise a nodule and look for adult worm or (very numerous) larvae. Clip off a bit of skin or conjunctiva, shake up in a few drops of salt solution and look for larvae. (Larvae are not present in the blood.) Complement fixation test positive with special antigens.

Ophthalmia Neonatorum.—(See Gonococcus Infections.)

Oriental Sore.—Examine scrapings from base of ulcer for Leishman-Donovan bodies. Stain with Wright or Giemsa. Preferably obtain material by aspiration by puncture near edge of ulcer. If bacterial contamination can be avoided, culture on N.N.N. medium. In espundia obtain material also from adjacent lymph nodes by puncture.

Oroya Fever.—Acute, rapidly developing haemolytic anaemia. Rod-like organism, Bartonella bacilliformis, in red cells.

Osteomalacia.—Either blood calcium or phosphorus (or both) usually decreased. Blood phosphatase increased. Roentgenogram.

Otitis Media.—Neutrophilic leukocytosis. Make smears from discharge and culture on blood agar. Make blood culture.

Pancreatic Disorders.—Examine faeces for undigested starch, muscle fibres, and abnormal amounts of total fat and neutral fat. Examine duodenal contents, especially for enzymes. (See p. 759.) Test urine for sugar. Determine blood sugar and diastase. Increased diastase in urine. Make glucose tolerance test.

Paragonimiasis.—Examine fresh sputum for light-yellow, operculated ova, averaging 90 by  $65\mu$ . Also for pus, blood, elastic fibres. Exclude tuberculosis.

Paratyphoid Fever.—Examine as for typhoid fever.

Pertussis.—In early stages: Examine stained smears from sputum for small, oval, Gram-negative, bipolar-staining bacilli. Culture sputum (or use "cough plate" method) on Bordet-Gengou medium. Usually a very marked lymphocytosis.

Piedra.—Examine hairs for small gritty masses which consist of spores arranged like mosaics about hairs.

Pituitary Disorders.—In hyperpituitarism (acromegaly) there may be polyuria and glycosuria associated with hyperglycaemia and reduced glucose tolerance. In hypopituitarism (dystrophia adiposo-genitalis, Froehlich's syndrome) blood sugar is low, glucose tolerance increased. Basal metabolic rate slightly retarded (but normal if calculated on basis of the ideal weight of the individual).

Plague—Bubonic Type.—Examine material obtained by gland puncture for *P. pestis*. Stain smears, culture, and inoculate a mouse or guinea pig to identify. *Pneumonic type:* Examine the thin, watery, blood-tinged sputum in the same way. To obtain pure culture, inoculate on unbroken skin or nasal mucosa of animal. *Septicaemic type:* Make blood culture. *P. pestis* may be sufficiently numerous to be found in blood films. Leukocytosis. Differentiate from tularaemia.

Pleural Fluid.—Determine specific gravity, albumin content, total cell count. Centrifugalize a small portion collected in citrated salt solution, treat sediment with 1% formalin, centrifuge again, and stain sediment by Gram's and Ziehl-Neelsen stains for bacteria and by Wright's stain for differential count. Culture on blood agar and inoculate a guinea pig. In tuberculosis the fluid usually shows a lymphocytosis. (See p. 643.)

Bronchopneumonia.—Culture sputum on blood agar. Make blood culture.

Pneumonia, Acute Lobar.—Isolate pneumococcus from sputum (or material from lung puncture) by culture and mouse inoculation. Determine type directly from sputum by Neufeld's "Quellung" reaction, or by precipitin tests on sputum or urine; confirm with culture later. Make blood culture (often positive early; late in disease indicates a bad prognosis). Marked neutrophilic leukocytosis with shift to the left, and reduction in cosinophiles. Low count indicates poor resistance. Urine chlorides much reduced. Blood chlorides reduced, non-protein nitrogen high if oliguria is marked. Administration of serum may be guided by agglutination tests with patient's serum. (See p. 41.)

Poliomyelitis.—Examine spinal fluid. (See p. 629.) Culture on blood agar to exclude ordinary forms of meningitis. Moderate neutrophilic leukocytosis at onset.

Polycythaemia.—High red cell count (over six million) with increased blood volume. Increased viscosity. Evidences of active red cell regeneration, neutrophilic leukocytosis with often a few myelocytes, and increased platelets. High blood calcium. Basal metabolic rate often accelerated. In selected cases test renal function and liver function.

Pregnancy. -Increased sedimentation rate. Friedman or Aschheim-Zoudek test positive. (See p. 785.)

Pregnancy, Toxaemia of.—Examine urine for evidences of nephritis. Test for ketone bodies, ratio of NH<sub>3</sub>/urea and other evidences of acidosis. Tests for renal function and liver function.

Prostatic Hypertrophy.—Examine urine for albumin, casts and evidences of infection. Make culture. Test non-protein nitrogen or urea and creatinin in blood. Usual tests of renal function not applicable except with catheterization.

Purpura Haemorrhagica (Thrombocytopenic).—Platelet count reduced. Bleeding time prolonged. Clot retraction impaired. Tourniquet test positive. Coagulation time usually normal. May show secondary posthaemorrhagic anaemia. Leukocyte count variable.

Pyelitis .- (See Urinary Tract Infections.)

Pyloric Obstruction.—Examine fasting stomach contents for evidences of retention. Roentgenogram. Oliguria. Dehydration, with high red cell volume, high plasma proteins. Low blood chlorides and bases, and high non-protein nitrogen and urea (normal creatinin), falling rapidly if diuresis is established. If HCl is vomited, alkalosis with high plasma bicarbonate and tetany. See Gastric Ulcer, Carcinoma of Stomach.)

Rabies.—Keep dog, which has bitten patient, alive to observe symptoms. If dog has been killed, make smears from cornu Ammonis and stain by Giemsa's or Mann's stain for Negri bodies. (See p. 185.)

Relapsing Fever.—Examine blood for spirochaetes with dark field or India ink method, or in smears stained by Wright's stain. (They may be absent from peripheral blood during afebrile period.) If not found, inoculate a mouse and examine its blood after 24 and 48 hours. Neutrophilic leukocytosis in acute cases. Differentiate from malaria, yellow fever, Weil's disease.

**Rickets.**—Blood phosphorus usually low, calcium normal. In some cases calcium is low and phosphorus normal. Product of  $Ca \times P$  is below 40 and often below 30. Blood phosphatase increased. Examine blood for anaemia. Roentgenogram.

Rickettsial Infections.—Inoculate nearly-grown male guinea pig intraperitoneally with 1 cc. of blood obtained during febrile period. Take temperature of pig regularly and watch for febrile reaction after 5 to 12 days. Watch for swelling of scrotim, and examine scrapings from tunica vaginalis for Rickettsiae (Mooser bodies). Examine brain sections for small proliferative nodules and perivascular infiltrations. (See Table, p. 23.) Test serum of patient after 7 days for agglutinins for Proteus O X 19 (Weil-Felix). Moderate neutrophilic leukocytosis.

Rocky Mountain Spotted Fever.—(See Rickettsial Infections.)

Scabies.—With the aid of a hand lens examine the infected skin for a black line which marks the tunnel for the parasite. The female can be found at the end of the tunnel and removed. If parasite is not found, look for ova in scrapings from skin.

Scarlet Fever.—Neutrophilic leukocytosis of 12,000 to 15,000 with early increase in eosinophiles. Make culture from throat on blood agar for haemolytic streptococci. Test for Schultz-Charlton reaction. Watch urine for evidences of nephritis. Make Dick tests on contacts.

Schistosomiasis.—Examine urine and faeces for ova, particularly in masses of blood-tinged mucus. Blood in urine. Examine blood for anaemia and eosinophilia. In late stages test liver function. Complement fixation reaction positive with special antigen.

**Screw-worm Infection.**—To identify, examine breathing slits on posterior stigmata of larvae found in auditory canal or skin ulcers.

Scurvy.—Tourniquet test of capillary resistance positive (as in purpura). Reduced excretion of cevitamic acid in the urine. May cause hypochromic anaemia.

Septicaemia.—Make blood culture. Neutrophilic leukocytosis with shift to the left.

Small Pox.—Initial leukopenia followed by neutrophilic leukocytosis in pustular stage. Monocytes increased. Try Paul's or McKinnon's inoculation tests. (See p. 174.)

Sprue.—Examine the frothy, pultaceous stools for undigested food and excess fat (25% to 40%), chiefly fatty acids. Make gastric analysis (occasionally an achlorhydria). Examine blood for anaemia, usually macrocytic, like pernicious anaemia;

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occasionally hypochromic. Blood calcium reduced. Glucose tolerance curve has flat peak.

Sporotrichosis.—Culture on agar or potato for 8 days or more. Direct smears do not show organisms.

Syphilis. - Primary stage: Look for T. pallidum in serous exudate from chancre in dark-field preparations (or stained films). If negative, and ulcer is healing, examine juice aspirated from regional bubo.

Secondary, tertiary and latent cases: Make Wassermann reaction or flocculation tests. Examine spinal fluid if clinical evidence of disease of the nervous system is present; and in all cases before treatment is stopped.

Tetanus.—Inoculate white mouse or guinea pig and make anaerobic cultures from curettings from the wound. (See p. 68.) Rarely found in smears. Inject filtrate from culture into two guinea pigs, one of which should be protected by injection of antitetanic serum.

Thrombocytopenia.—(See Purpura, thrombocytopenic.)

Thrush.—Make scrapings from lesions and examine in 10% KOH solution. The organism, Syringospora albicans, may be cultivated on Sabouraud's medium. It slowly liquefies gelatin and blood serum and acidifies and clots milk. In cultures there are budding yeast-like forms and mycelial threads.

Thyroid Disorders.—See Exophthalmic goiter and Myxoedema.

Transfusion.—To select donor, secure individuals of the same blood group (or group O), and match the serum of the recipient with the cells of the donor and vice versa. Exclude syphilis by Wassermann or Kahn test and by physical examination. Exclude malaria by history and stained thick blood films.

Trichiniasis.—Usually high leukocytosis and eosinophilia. Secure suspected meat, examine for encysted larvae in press preparations, or digest in artificial gastric juice, and collect larvae in Baermann apparatus. (See p. 494.) May feed meat to rat or mouse and examine muscles similarly after 10 days. During second or third week take 5 to 10 cc. of blood in dilute acetic acid and examine sediment for larvae. After second week excise a bit of muscle from deltoid or pectoralis near insertion and examine. Make intracutaneous test with Bachman antigen.

Trypanosomiasis.—Examine blood for trypanosomes in fresh preparations or stained thick films. When sparse, concentrate in blood by centrifugalization and make films from leukocyte layer. (See p. 426.) If not found, inoculate a rat or guinea pig intraperitoneally with blood, gland juice or emulsion of excised gland and examine blood at intervals. Formol-gel test often positive. In lethargic stage examine spinal fluid for parasites. Cell count and globulin increased. Differentiate from kala azar, malaria, syphilis.

Tuberculosis.—Make acid-fast stain of smears from sputum, faeces or urinary sediment. If necessary, first concentrate by digesting in alkali or antiformin and centrifugalizing. Culture on Petroff's medium or in Corper's medium. Inoculate guinea pig. Blood cultures may be positive in miliary tuberculosis. High monocyte-lymphocyte ratio indicates progressive lesion. Positive diazo-reaction in urine an unfavorable sign. In special cases make intracutaneous or subcutaneous tuberculin tests, or in children von Pirquet's cutaneous test. Sedimentation rate increased in proportion to activity of disease.

Tularaemia.—In the early stages inoculate mouse or guinea pig with material from local lesion or regional glands, or with blood. At autopsy look for characteristic lesions

(small caseous foci in organs) and make cultures from blood and organs on glucose cystin blood agar. After the first week test blood for agglutinins. Differentiate from Brucellosis, plague.

Typhoid Fever.—Neutrophilic leukopenia; relative lymphocytosis; cosinophiles reduced or absent. Blood culture usually positive during the first week, later less frequently obtained. Culture urine and faeces on Endo, Teague or bismuth sulphite medium. Make agglutination test after 7 to 10 days. In suspected carriers culture urine and faeces or duodenal contents. Differentiate from paratyphoid fever, brucellosis, malaria, typhus, miliary tuberculosis, liver abscess, kala azar.

Undulant Fever.—(See Brucellosis.)

Typhus Fever.—(See Rickettsial Diseases.)

Urinary Tract Infections.—Examine sediment immediately in hanging drop and in films stained by Gram. Collect specimen with sterile precautions or by catheter and culture on agar and blood agar plates. In special cases search for tubercle bacilli by stain and culture and confirm by guinea pig inoculation. Digest sediment in alkali if abundant or contaminated.

Yellow Fever.—Early neutrophilic leukocytosis which in a few days falls to normal or below. Increasing albuminuria with granular and epithelial casts from the first or second day. Oliguria or anuria in fatal cases. Bile pigments are present in blood and urine in increasing amounts from the second or third day. Inject blood of patient (during the first three days) intracerebrally into mice. Serum of cases after recovery shows life-long protective power. Differentiate from severe malaria, blackwater fever, infectious jaundice, relapsing fever, dengue, influenza.

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